An Assessment of the Differentiation Potential of Epithelial Stem Cells in Adult Endometrium

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree Master of Philosophy

by

Louise Marie Figueira da Silva

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Abstract

Background: We are at the beginning of a new era where regenerative medicine is really starting to take hold. An appreciation of adult stem cells and their role in tissue maintenance have heralded novel insight into endometrial remodelling. The idea that human endometrium could be a plentiful source of adult stem/progenitor cells (ASPCs) has captured the imagination of many stem cell biologists. Not only that, they are also hypothesised to be involved in endometrial proliferative diseases, such as endometriosis. However, work remains preliminary and is stunted by a lack of specific endometrial stem cell markers. Recently, stage specific embryonic antigen -1 (SSEA-1) was proposed as a candidate epithelial ASPC marker in adult endometrium. In order to validate this, stem cell assays are required. These assays include measurements of clonogenicity, prolonged self-renewal and differentiation potential.

Aim: To assess the differentiation potential of SSEA-1+ cells *in vitro*.

Methods: All endometrial culture samples were collected from pre-menopausal women undergoing surgery for benign disease at Liverpool Women's Hospital (LWH). Epithelial cells were cultured in 3D Matrigel^{TM} to produce gland-like structures. Gland-like structures were characterised via immuno-histochemistry, to validate their resemblance to glands in endometrial tissue *in vivo*. To assess multi-potency, magnetic cell sorted (MACS) SSEA-1 enriched and depleted cell fractions were cultured in adipogenic and osteogenic inducing media. In order to assess

pluripotency, SSEA-1 enriched and depleted fractions were cultured in neurogenic media.

Results: SSEA-1 enriched cell fractions had a greater propensity to produce gland-like structures in 3D culture than SSEA-1 depleted cell fractions. Gland-like structures best resembled glands in post-menopausal (PM) endometrium. Non-gland like structures also formed in 3D culture from endometriosis samples, which stained exclusively and intensely for CK5/6. SSEA-1 enriched and depleted fractions were unable to differentiate into other cell types of mesodermal lineage. However, both enriched and depleted fractions were able to produce ectodermal derived neural-like PGP9.5 positive cells.

Discussion: This work confirms SSEA-1 is a reliable epithelial ASPC marker in adult endometrium. SSEA-1+ cells possess an increased ability to produce gland-like structures, reminiscent of *in vivo* endometrial glands. This therefore confirms unipotency. The presence of CK5/6 in non-gland-like structures from endometriosis samples suggests a potential role in endometriosis aetiology, and warrants further work. SSEA-1 enriched and depleted cells were unable to undergo multi-lineage differentiation, but were capable of producing neural-like cells. As both cell populations were unable to demonstrate multipotency, it unlikely SSEA-1 enriched/depleted cells are pluripotent. More likely, a neural progenitor may exist in adult endometrium, one that has been included in both cell populations. Given the close proximity of endometrial glands to the placenta, the commitment of epithelial stem cells could be an ancillary mechanism to prevent maternal microchimersim in offspring, which is associated with a number of pathologies.

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8-OHdG	8-oxo-2'-deoxyguanosine
ABCG2	ATP binding cassette sub-family G member 2
ACTB	β-actin
ALP	Alkaline phosphatase
aP2	Adipocyte Protein 2
APES	3-aminopropyltrithoxysilane
ASPC	Adult Stem/Progenitor Cell
ASRM	American Society for Reproductive Medicine
α TTP	α-tocopherol transfer protein
β -GP	β-Glycerophosphate
BAX	Bcl-2-Associated X Protein
BM	Bone Marrow
BMDC	Bone Marrow Derived Cell
BMDSC	Bone Marrow Derived Stem Cell
BrdU	Bromodeoxyuridine
BSP	Bone Sialoprotein
BSO	Bilateral Salpingo-oopherectomy
C/EBPA	CCAAT-enhancer-binding proteins
cDNA	Complimentary Deoxyribonucleic Acid
CE	Cloning Efficiency
CK	Cytokeratin
CK18	Cytokeratin 18
CK5/6	Cytokeratin 5/6
COC	Combine Oral Contraceptive Pill
CPP32	Cystein Protease Protein-32
DAB	3,3-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease Enzyme
dNTP	Deoxynucleotide Triphosphates
dsDNA	Double Strand DNA
DTT	Dithiothreitol
ECM	Extra-cellular Matrix
EEp-ASPC	Endometrial Epithelial Adult Stem/Progenitor Cells
EGF	Epithelial Growth Factor
ER-α	Oestrogen Recepetor-α
ER-β	Oestrogen Receptor-β
ESC	Embryonic Stem Cell
ESt-ASPC	Endometrial Stromal Adult Stem/Progenitor Cell
ET-1	Endothelin -1
FACS	Fluorescence activated cell sorting
FBS	Foetal Bovine Serum
FDA	Food and Drug Association
FGF2	Fibroblast Growth Factor - 2
FISH	Fluorescence In Situ Hybridization

FITC	Fluorescein Isothiocyanate
FSH	Follicular Stimulating Hormone
FT	Full Thickness
GCP	Good Clinical Practice
GFP	Green Fluorescent Protein
GnRH	Gonadotrophin-Releasing Hormone
H_2AX	Histone H_2A
HIF	Hypoxia Inducing Factor
HLA	Human Leukocyte Antigen
hMSC	Human Mesenchymal Stem Cells
hMSCGM	Human Mesenchymal Stem Cell Growth Media
HRP	Horse Radish Peroxidase
IBMX	3-isobutyl-1-methylxanthine
IL	Interleukin
iPS	Induced Pluripotent Stem Cell
ITS	Insulin-Transferrin-Selenite
i.v.	intra venously
IVF	In Vitro Fertilisation
LH	Luteinizing Hormone
LIF	Leukemia Inhibitor Factor
LMP	Last Menstrual Period
LNG-IUS	Levenorgestrel Intrauterine Systemt
LPL	Lipoprotein lipase
LRC	Label Retaining Cell
LREC	Liverpool Research Ethics Comittee
LWH	Liverpool Women's Hospital
MACS	Magnetic Cell Sorting
Mc	Micro-chimerism
MIPS	Metastatic Inducing Proteins
MMc	Maternal Micro-chimerism
MMP	Matrix Metalloproteinases
MRI	Magnetic Resonance Imaging
MSC	Mesenchymal Stem Cell
NAD	Nothing Abnormal Detected
NBF	Neutral Buffered Formalin
NSAIDS	Non-Steroidal Anti-Inflammatories
OC	Osteocalcin
OCT4	Octamer-binding transcription factor 4
OSX	Osterix
PBS	Phospho-Buffered Saline
PCR	Polymerase Chain Reaction
PDGF-R β	Platelet Derived Growth Factor – Receptor-β
PFA	Paraformaldehyde
PI	Propodium Iodide
PKA	Protein Kinase A
PM	Post-Menopausal
PODXL	Podocalyxin-Like Protein
PPARy2	Peroxisme Proliferator-Activates Receptor Gamma
PPH3	Phospho-Histone H3
PR	Progesterone Receptor

qPCR	Quantitative Polymerase Chain Reaction
REST	Relative Expression Software Tool
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription –Polymerase Chain Reaction
Runx2	Runt-Related Transcription Factor 2
s.c.	subcutaneously
SC	Stem Cell
SP	Side Population
SSEA-1	Stage Specific Embryonic Antigen -1
TA	Transit Amplifying Cell
TBS	Tris-Buffered Saline
TGF- β 3	Transforming Growth Factor-β3
TIMP	Tissue Inhibitor of Metalloproteinases
TVS	Trans-Vaginal Sonography
uNK	Uterine Natural Killer Cells
VSEL-SCs	Very Small Embryonic Like – Stem Cells
WOI	Window of Implantation

Chapter One: INTRODUCTION

The organised regenerative ability of human endometrium is unmatched by any other organ. Under the influence of ovarian hormones, the uterine lining undergoes a monthly cycle of proliferation, differentiation and shedding. Conclusive work has highlighted mechanisms underpinning endometrial shedding, yet there persists a void when trying to understand endometrial re-growth. A new era of regenerative medicine, and an appreciation of stem cells have heralded novel insight into endometrial expansion; it is likely stem cells are implicated in endometrial proliferation and proliferative disorders. In order to evaluate the possibility of endometrial stem cells in health and disease, a thorough literature review on the subject will be discussed.

1.1: Endometrial Structure and Function

1.1.1: Human uterus

The human uterus is a hollow muscular pear shaped organ, approximately 7.5cm in length. It serves to act as a conduit for the migrating sperm, and houses the development of the embryo and foetus. It is arguably the most plastic organ, in terms of tissue remodelling.[1]

The uterus consists of the fundus superiorly, the corpus, and the cervix inferiorly. It has three openings; the two fallopian tubes and the external os, which connects the uterus with the vagina. Structurally, the uterus is covered with peritoneum and

contains a thick muscular layer (myometrium), made up of a criss-cross of involuntary fibres mixed with fibro-elastic connective tissue (Figure 1.1). The inner lining of the uterus is termed the endometrium. Human endometrium serves three main functions; to maintain the patency of the uterus by preventing adhesions from surrounding myometrium; to provide an optimum environment for potential implantation of a blastocyst; and should implantation occur, it forms the decidua in pregnancy.

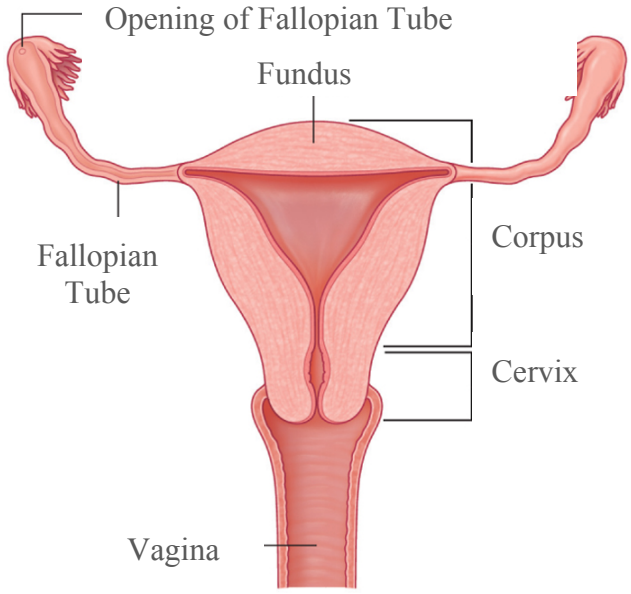

Figure 1.1: Coronal cross-section of human uterus and vagina. Schematic diagram demonstrating anatomical composition of the human female reproductive organs and divisions of the human uterus, adapted from *Drake et al.*[2]

1.1.2: Human endometrial composition

Histologically, endometrium differs on account of its location within the uterus. Endometrium is present in the fallopian tubes and uterine corpus. The cervical canal does not contain endometrium; the endocervix is made up of tall columnar cells, which form a series of complicated branching glands. It is these glands that secrete the 'cervical plug', an alkaline mucus, which forms a protective barrier.[3] The endometrium of the fallopian tubes contains columnar ciliated cells. The purpose of

this is to aid in transportation of the zygote to the corpus. The endometrium of the corpus is made up of a single layer of ciliated epithelial cells, which rest on a bed of connective tissue. The connective tissue is formed by a pool of stromal cells, which harbour a rich blood supply in the form of spiral arterioles, along with leucocytes and nerve fibres. Simple tubular epithelial glands dip down from the luminal edge to the base of the stroma. It is the endometrium of the uterine corpus that undergoes extensive changes during the menstrual cycle and shall be discussed in detail throughout this chapter.

Endometrium of the uterine corpus varies in thickness, ranging from 1mm during menstruation up to 10mm in the secretory phase.[4] It can be divided into two main layers; the basalis/basal layer and the functionalis/functional layer (Figure 1.2). The basal layer is adjacent to the myometrium and remains constant throughout a woman's life. It comprises dense stroma, large blood vessels and glandular bases. It is the basal layer that generates the transient functionalis each month. The functionalis is adjacent to the uterine cavity, and undergoes partial/complete disintegration and regeneration in response to oestrogen and progesterone. The purpose of remodelling is to provide an optimum environment conductive to implantation of the conceptus, but only when appropriate.[5,6]

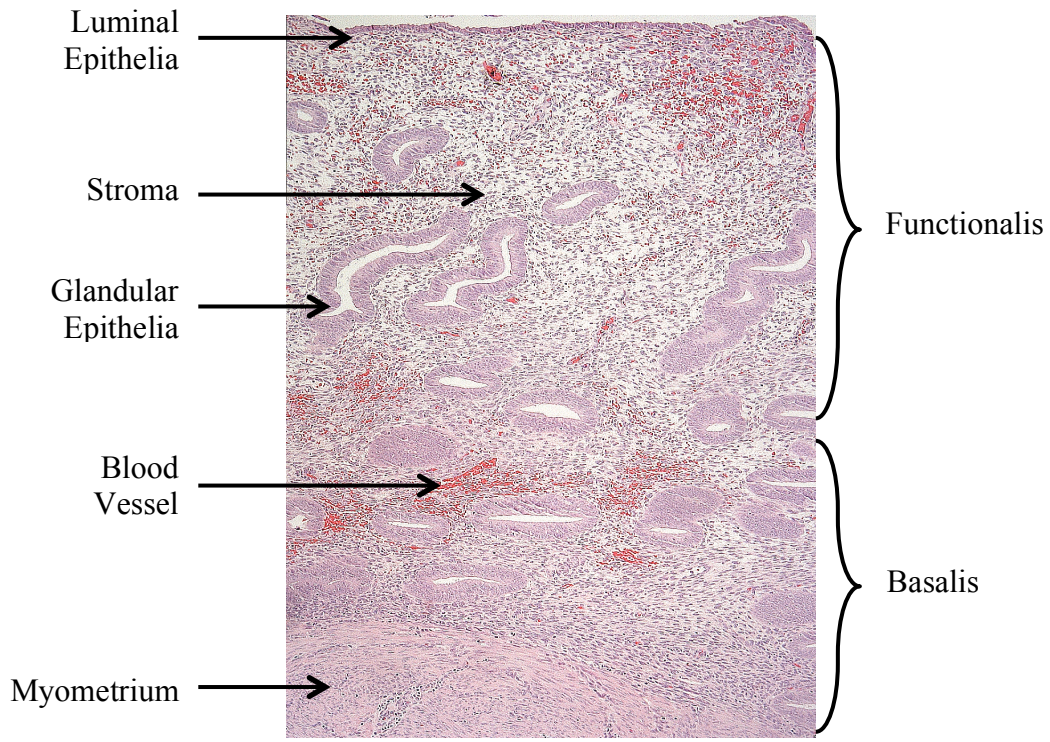

Figure 1.2: Micrograph of full thickness human endometrium. Haematoxylin and Eosin staining of a full thickness section of human endometrium.

1.1.2i: Epithelia

Epithelia are continuous sheets of tightly linked cells that constitute mucosal surfaces and linings. They form the parenchyma of most tissues. Epithelia can be simple, stratified or 'pseudo-stratified'. They possess an elegant intrinsic scaffold that maintains cell polarity, provides anchorage to the basement membrane, and facilitates important inter-cellular communications. In culture, epithelia aggregate in clusters and are typically polygonal in shape. The presence of cytokeratin (CK) is definitive of epithelia. They can be either luminal; lining cavities, or glandular; forming endocrine or exocrine glands. Luminal epithelia have a particularly important function in the endometrium; apposing and adhering to the implanting blastocyst. Glandular epithelia sustain the trophoblast during the first-trimester, as maternal circulation to the human placenta is not established until 10-12 weeks.[7]

They secrete a cocktail of growth factors including alpha tocopherol transfer protein (αTTP), transforming growth factor (TGF-β_3) and leukaemia inhibiting factor (LIF).[8]

1.1.2ii: Stroma

Endometrial mesenchyme harbours a collection of stromal cells, blood vessels, nerve fibres and leukocytes. Stroma are undifferentiated cells that have the ability to migrate in tissue. As epithelial cells are avascular, stromal cells play a supportive role; providing the right environment to permit the epithelial cells to function. Stroma form the extra-cellular matrix (ECM), a complex pool consisting of collagens, fibronectin, glucose and growth factors. Most endometrial stromal cells resemble undifferentiated fibroblasts, which under the influence of progesterone engorge to become 'pre-decidua' in the late secretory phase and decidua in pregnancy.[9] More recently, work has shown the stromal cells are responsible for the cascade of reactions leading to endometrial breakdown.[10]

1.1.2iii: Leucocytes

Leucocytes are heavily influential in the remodelling of endometrium. They account for 7% of the stromal fraction in the proliferative phase, and this greatly increases to 40% at the time of menstruation.[9,11] Resident lympho-myeloid cells consist of T lymphocytes, macrophages and uterine natural killer cells (uNK). T lymphocytes are located in lymphoid aggregates at the base of glands or intra-epithelialy. Macrophages and uNK both increase during the mid to late-secretory phase and are speculated to have a role in angiogenesis.[12]

1.1.2iv: Endothelia

The uterine and ovarian arteries provide the blood supply to the endometrium. Radial arteries form in the myometrium, crossing the endometrial border to become anastomosing basal arteries in the basalis. These branch off to become terminal spiral arteries in the functionalis and form a sub-epithelial plexus under the luminal epithelia. Endometrial endothelia are not immune to the cyclical changes of the menstrual cycle. The process of endothelial regeneration is complex, and will only be discussed very briefly here. Angiogenesis dominates vessel formation; vessel elongation is a major mechanism in the late proliferative phase, followed by a stage of remodelling via vessel intussusception.[13,14]

1.1.2v: Nerve fibres

Neurons are specialised cells that are electrically excitable. Work to establish the presence of neural fibres in normal human endometrium is lacking. One study demonstrated the presence of neural fibres in the basal third of human endometrium but these were lost in the functional layer.[15] Presumably, neurons within the endometrium do not undergo cyclical regeneration.

1.1.3: The menstrual cycle

The menstrual cycle is present in humans, primates and the elephant shrew.[16] The reason behind menstruation is unclear but there is a link between mammals which menstruate and those who exhibit haemo-chorial placentation (maternal blood comes into direct contact with the foetal chorion).[17] The reason for menstruation is most likely to improve nutrition for the potential blastocyte. The blood supply to the placenta does not arise until 12 weeks gestation; until this period the decidua is

responsible for nutritional support.[7] During the cycle, stroma undergo priming to improve nutritional status in the form of 'decidualisation'. This is a non-reversible transformation and therefore if implantation does not occur, these cells must be discarded and a replacement formed.[18]

The menstrual cycle is described as 28 days in length. It comprises the uterine and ovarian cycle: the two run concomitantly, and the ovarian cycle plays a critical role in regulating its uterine counterpart. The menstrual cycle is divided into three phases; menstrual (day 1-4), proliferative/follicular (day 5-14) and secretory/luteal (day 15-28). The cycle relies on negative feedback of oestrogen and progesterone on gonadotrophin releasing hormone (GnRH) and the pituitary hormones; follicular stimulating hormone (FSH) and luteinizing hormone (LH).

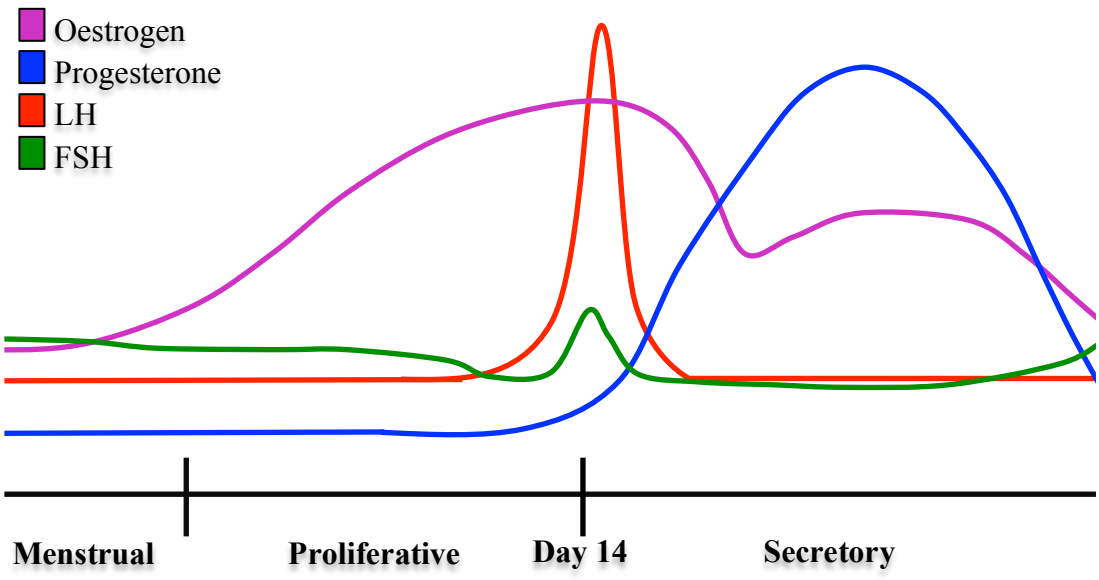

Figure 1.3: Hormonal changes in the menstrual cycle. The cycle is divided into three phases. Oestrogen levels gradually increase during the proliferative phase, resulting in an LH spike at day 14. Once ovulation has occurred, progesterone levels increase in the secretory phase. If fertilisation does not take place, the cycle is repeated and menstruation ensues.

1.1.3i: The ovarian cycle

Human ovaries are the female gonads, existing in pairs and attached to the uterus via the ovarian ligaments. They serve to produce ova, a process known as oogenesis, and secrete the female sex steroids oestrogen and progesterone. Oogenesis is a long and intermittent process. Primitive precursors (oogonia) initially undergo mitosis and maturation, during the third trimester of foetal life, to become primary oocytes. At birth, it is estimated a female possesses 2×10^6 primary oocytes.[19] The primary oocytes (diploid cells) begin the first stage of meiosis shortly after parturition. However, development is arrested in prophase I until puberty. At this point, growth and development is independent of GnRH.[20] After puberty, a primary oocyte completes meiosis I prior to ovulation. This produces a secondary oocyte and a small polar body. Only 400 primary oocytes reach this stage; the rest undergo atresia.[19] Meiosis II only is completed if fertilisation occurs, resulting in an ovum and a second polar body.

Follicles are the basic units of the ovary and house the developing oocyte. These also undergo a maturation process similar to the oocyte. Primoridal follicles form *in utero* and house the oogonia. From birth onwards, primordial follicles mature to become primary follicles, producing the mucopolysaccharide zona pellucida, around the oocyte, and developing granulosa and theca cells. The ovarian cycle has two phases: follicular and luteal.

Follicular phase

At the beginning of the cycle, oestrogen and progesterone levels are low. Due to negative feedback, GnRH is released from the hypothalamus causing FSH and LH to

be released from the anterior pituitary.[21] As the name would suggest, FSH encourages follicular growth; theca cells increase in number and differentiate to become the theca interna and externa of the ovum. Theca cells also recruit androgens and convert into oestrogen. Granulosa cells too increase in number and secrete a serous fluid, causing the follicle to engorge so that it becomes grossly visible on the ovarian surface. The increase in intra-follicular pressure makes it prone to rupture. Oestrogen levels continue to rise and would normally produce a negative feedback effect. However, when oestrogen levels rise above to a significant level, a positive feedback effect is observed and produces an LH surge, ultimately leading to ovulation.[21]

Luteal phase

During ovulation the primary follicle ruptures and is released from the ovary. It produces two products; the secondary oocyte (surrounded by the zona pellucida and granulosa) and follicular remnants. Under the influence of LH, follicular remnants develop into the corpus luteum, which is the source of progesterone. The corpus luteum has a fixed life span of 12-14 days if implantation does not occur and thereafter it undergoes apoptosis; progesterone levels decrease and the cycle is repeated unless pregnancy is established.[22]

1.1.3ii: The uterine cycle

The uterine cycle describes the morphological changes and remodelling of the endometrium. As mentioned earlier, it runs concomitantly with the ovarian cycle, and is controlled largely by ovarian hormones. The follicular phase of the ovarian

cycle correlates with the menstrual and proliferative phase of the uterine cycle. Similarly the luteal phase is representative of the secretory stage.

Menstrual phase

Day 1 of the cycle is considered to be the first day of menstruation and is the result of progesterone withdrawal. There has been considerable controversy regarding the believed mechanism underpinning menstruation. *Markee et al.* initially proposed hypoxia was the driving force, after a series of experiments transplanting endometria into the eyes of rhesus monkeys.[23] However, there has since been a shift in thinking. *Hisaw and Hisaw* in 1961 demonstrated menstruation occurred even in the presence of atrophy.[24] In addition, hypoxia inducing transcription factors-1α (HIF-1α) and HIF-2α, are barely detectable within the endometrium in the peri-menstrual phase.[25]

The most accepted dogma proposes that matrix metalloproteinases (MMPs) are key players in initial *focal* breakdown.[10,26] MMPs are secreted in latent forms by endometrial stromal cells, and are activated either by other MMPs (mainly MMP-3 and MMP-9) or products of leucocytes. Studies have demonstrated MMP inhibitors can stop the breakdown of endometrial explants in culture.[27] Natural tissue MMP inhibitors (TIMPs) have also been found within the endometrium, indicating there is a delicate interplay between tissue breakdown and maintenance.[28]

There is also evidence to suggest that menstruation is the consequence of an immune reaction. *Kelly et al.* demonstrated that progesterone negatively regulates IL-8 expression in endometrium, resulting in leukocyte migration.[29] Leucocytes produce enzymes, namely elastase and tryptase, converting latent MMPs into their active

counterpart. Once this takes place, a cascade of MMP reactions occurs. The resulting fragmentation leads to loss of blood vessel integrity and hypoxia shortly ensues, causing rapid degeneration of the functional layer and blood loss.[9] Ancillary mechanisms exist to restrict loss; Endothelin 1 (ET-1) has been shown to be important at controlling blood loss, whereas TIMPs are activated to restrict endometrial loss.[30] Scanning electron microscopy has revealed that small lesions are apparent as early as the late secretory phase, supporting that rapid endometrial dissolution is a consequence of *initial focal* breakdown.[31]

Apoptotic bodies are also found within the endometrium, interestingly most are found within glandular epithelia and very low levels are observed in stroma. Reports have shown Bcl-2-associated X protein (BAX), a pro-apoptotic protein initiating mitochondrial permeablization, to be poorly expressed in proliferative epithelia but high in secretory epithelia.[32] The reverse pattern is observed with Bcl-2, an anti-apoptotic marker.[32,33] Similarly, 'death receptors' Fas and Fas/L are present throughout the cycle but inactive. It is only during the late secretory phase, under the influence of MMPs, they become active via incorporation into the cellular membrane.[33] Therefore, it would seem menstruation is an interplay between an inflammatory response, hypoxia and apoptosis.

Proliferative phase

The proliferative phase follows menses and involves priming of the endometrium as a response to oestrogen. The length of this phase can vary from 9-23 days.[34] Morphologically, the endometrium becomes thicker due to acquisition of the functionalis, increasing in thickness to 4mm.[4] Glands begin as fairly undifferentiated,

short and straight, with a circular cross-section, but by the end of the phase functional glands are long and tortuous. Differences of epithelia are also observed on a cellular level. Glandular epithelia gradually increase in length to become tall and columnar. Pseudo-stratification increases, reaching a maximum at day 10 in a 28 day cycle.[35] Although increased mitotic activity, stroma undergo very little differentiation during this phase. Interestingly, scanning microscopy has revealed a shared origin for re-epithelialisation. Outgrowth from exposed basal glands runs alongside ingrowth from the peripheral surface membrane; stroma in the functionalis is only formed once the endometrium has been completely re-epithelialised.[36]

Secretory phase

After the re-growth of the functionalis, the influence of progesterone brings about differentiation. The secretory phase lasts 14 days, allowing for little variation. The stage aims to create an optimum environment for embryo implantation. However, early and late secretory endometria are still non-responsive to the conceptus; there is only a very small timeframe in which the endometrium is receptive. In 1973, *Psychoyos* coined the term 'Window of Implantation' (WOI) to describe days 20-24 (in a 28 day cycle).[37] This can be regarded as the most differentiated form the endometrium takes; the only time when it is receptive to the blastocyst. This is believed to be a survival mechanism and only permits implantation of the blastocyst at the correct stage of development.[5,6]

It is now that glandular activity is at its highest. The collection of secretions is termed the histotroph and is the subject of much research. Glandular epithelia lose their pseudo-stratified appearance; sub-nuclear vacuoles push the nuclei to the apical

surface.[4] Gland luminal diameter also reaches its maximum, causing glands to occupy nearly a quarter of the endometrium.[4]

One key feature of luminal epithelia is its ability to accept/reject a blastocyst – it is during the WOI that this characteristic now comes into play. By day 20 (of a 28 day cycle), flattened 'droplet like' projections appear on the luminal surface.[38] These deposits, called pinopodes/uterodomes, are believed to be 'landing platforms' for the potential blastocyst and disappear within 4 days of formation.[39] Other biochemical changes have been documented; Muc-1, a mucin thought to cause steric hindrance between blastocyst and luminal apical surfaces is locally down-regulated at implantation sites in humans.[40]

During the last week of the cycle, glandular secretions cease and glands take on a characteristic serrated appearance. At this point, it is the stromal population that takes centre stage; stromal phenotype dramatically changes from undifferentiated fibroblast to something that best resembles epithelia. Under the influence of progesterone, stroma engorge, accumulate glycogen and become secretory.[4] If implantation occurs, this phenotype continues into pregnancy.

1.1.4: Hormone receptors

Expression of the hormone receptors also alters throughout the course of the cycle and will only be discussed briefly here. As expected, oestrogen receptor (ER) is greater expressed in the proliferative phase. Two forms of oestrogen receptor exist; ER-α and ER-β. ER-α is present in both stroma and epithelia of the functionalis. ER-β is not detected in the stroma but only this form is present in vascular endothelia.[9]

Knockout studies in mice would suggest ER-α is crucial at preserving fertility whereas ER-β is dispensable.[41] Similarly, two forms of PR also co-exist, PR-A and PR-B.

1.1.5: Endometrial morphogenesis

The female reproductive organs are formed from the mesonephric (Wolffian) and paramesonephic (Müllerian) ducts of intermediate mesoderm. Until the seventh gestational week, sex of the foetus is indifferent as both ductal systems are present. The two pairs of ducts are arranged in tandem, the Müllerian duct arising from invaginations in the abdominal cavity to cross the mesonephric ducts medially (Figure 1.3). During the seventh week of gestation, the lower portions of the Müllerian ducts fuse in females, to form the uterine canal.[1] The upper portions remain distinct and form the Fallopian tubes. At the same time, the Wolffian ducts undergo atrophy in females.

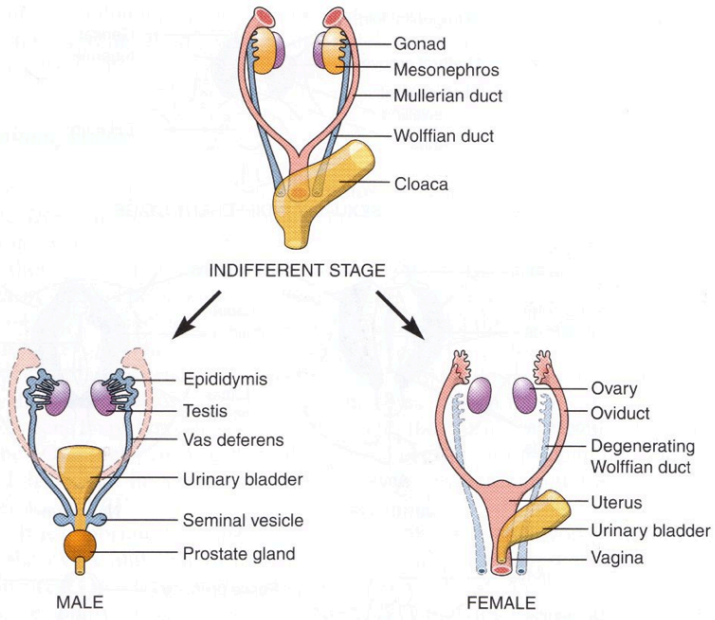

Figure 1.4: Organogenesis of the male and female reproductive organs.[42] Müllerian ducts fuse in the female to become the uterine canal at the 7th week of gestation. Wolffian ducts atrophy.

Formation of the endometrium itself is initiated by the differentiation of the Müllerian duct mesenchyme.[43] It is at this point that adenogenesis begins. Simple luminal columnar epithelia invaginate to produce buds (Figure 1.4). Once bud formation has occurred, tubulogenesis ensues; cellular divisions produce a tube-like structure. Subsequent branching and coiling follows, extending deeper into the endometrium, until reaching the myometrial junction.

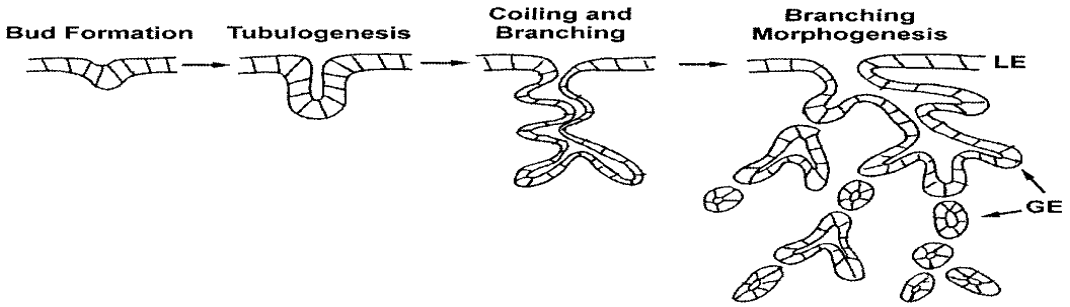

Figure 1.5: Schematic diagram of adenogenesis. Adenogenesis occurs in four steps: 1) bud formation; 2) tubulogenesis; 3) coiling and branching; 4) branching morphogenesis.[43]

The process is long and only complete at puberty. Neonatal endometrium is underdeveloped; luminal epithelium is present with few sparse glands in superficial areas. By 6 years of age, endometrial glands have migrated only half-way through the endometrium, to reach their end-point in the basal layer at sexual maturation.[44] The migratory ability of epithelia during adenogenesis suggests there are changes in the ECM, basement membrane, and mesenchyme-epithelia interactions.[43] It is important to note the development of endometrial glands arises from the luminal surface, however their monthly replenishment is a result of basal glandular activity.

1.1.6: Endometrium conclusion

The mucosal lining of the uterus is heterogeneous, consisting of epithelia, stroma and leucocytes. Each month, human endometrium undergoes differentiation to provide an

optimum habitat for implantation and blastocyst development. The differentiation is non-reversible. If implantation does not occur, a well-known cascade of reactions results in menstruation. However, it is the successive proliferative abilities of the endometrium that are less well understood; such rapid re-growth would suggest the existence of stem cells.

1.2: Endometriosis

Endometriosis is a benign, chronic disorder defined by abnormal growth of endometrium outside of the uterine cavity.[45] The disease was first defined by the general surgeon Thomas Cullen.[46] He recognized the two key features: i) the presence of endometrial glands and stroma outside of the uterine cavity and ii) ectopic endometrial glands and stroma were benign and non-neoplastic in nature.

1.2.1: Epidemiology

Endometriosis is a significant cause of female infertility and morbidity. A surgical examination is required for diagnosis, and for that reason the true prevalence remains controversial. In asymptomatic women undergoing tubal ligation, the prevalence is thought to be about 4%, (range 1-7%).[47] The disease should be suspected in women suffering from pelvic pain and primary infertility, present in 50%-60% and 17% respectively.[48,49] On average it is estimated 10% of reproductive women suffer from endometriosis, whether the disease is symptomatic or not.[50]

1.2.2: Clinical features

Endometriosis paints a variable clinical picture and clinical features rely largely on position of ectopic lesions. Typically, the presenting complaint is pain. This is usually chronic, presenting itself in the form of dysmenorrhoea or dyspareunia. Pain can be cyclical or continuous, and dull or throbbing. Patients commonly report a burning or hypersensitivity, suggesting a neuropathic component.[51] Pain is often accompanied by bowel symptoms such as dyschezia. However, patients can be asymptomatic and the presenting complaint may be a difficulty to conceive. A bimanual examination usually reveals focal pain/tenderness and/or a fixed retro-

verted uterus. Due to the variation of presentation, there is considerable overlap with other gynaecological and non-gynaecological diseases such as inflammatory bowel disease and depression, making diagnosis challenging. On average it is estimated the time from initial symptoms to diagnosis is over ten years.[52]

1.2.3: Investigations

Non-invasive investigations such as magnetic resonance imaging (MRI) and trans-vaginal ultrasound (TVS) have a 80-90% sensitivity and 60-98% specificity at detecting ovarian endometriomas but results are less impressive when detecting lesions at other locations.[53] Ca-125 can be elevated but again this has poor sensitivity and specificity.[54] Surgical visualisation followed by histological confirmation remains the gold standard.[55]

1.2.4: Pathology and staging

Endometriosis is an oestrogen dependant disease; lesions consequently wane throughout pregnancy, menopause and menstruation. Conversely, there are reports of endometriosis being present in males, and women suffering from Turner's syndrome.[56,57] However, in both these cases patients were taking exogenous hormones, and thus enabled ectopic growth. Ectopic lesions are commonly found on the ovaries, fallopian tubes, pelvic peritoneum and bladder. Lesions can be observed in distant organs such as the lung, although admittedly these cases are rare.[58] Within endometriosis, three 'sub-diseases' are thought to exist: peritoneal, ovarian and rectovaginal.[59] Red lesions, 'powder-burn', white lesions, 'chocolate cysts' and microscopic lesions, are all descriptors of this disease's variable form. Lesions comprise endometrial glands and stroma, although appearance is somewhat

dissimilar to eutopic tissue (Figure 1.5). Ectopic glands are usually less organised and less differentiated. Morphology and histochemical data suggest 'red' lesions best resemble proliferative eutopic endometrium, proposing these represent early implantation and disease.[59] Irritation of 'menstruating' red lesions leads to an inflammatory response within the pelvic cavity, producing fibrosis, which ultimately leads to adhesions and distortion of pelvic anatomical structures. Ovarian endometriosis typically produces cysts in the form of endometriomas, and recto-vaginal lesions are rich in smooth muscle with sparse stroma.

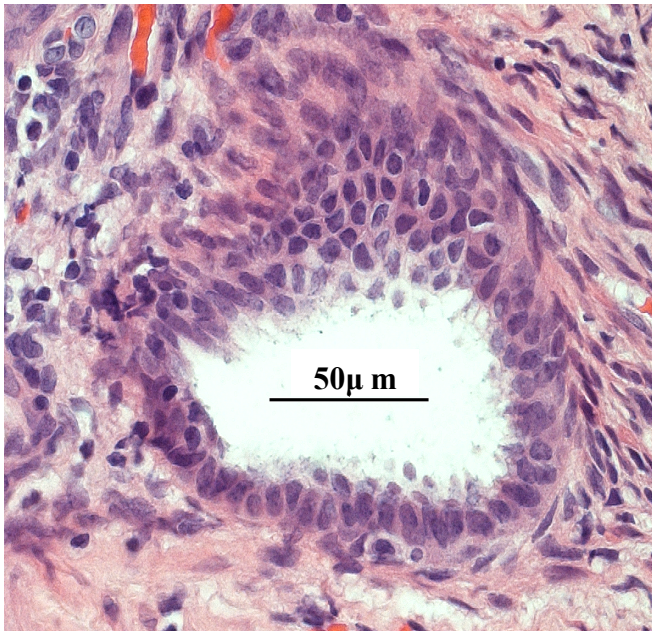

Figure 1.6: Ectopic endometriotic gland and stroma. Haemotoxylin and Eosin staining of a well differentiated ectopic gland, consisting of endometrial epithelia and stroma.

Histological analysis suggests that increased angiogenesis and neural infiltration are also significant features of endometriosis. An Australian group discovered neurons are increased in eutopic tissue from women with endometriosis, compared to those without.[60] This could be the reason why pain is the dominant symptom, and is consistent with other work.[61-63] The reasons and mechanisms behind increased angio- and neurogenesis remain unclear. There are reports of increased angiogenic and

neural growth factors in the pelvic fluid of women with endometriosis compared to those without. This has resulted in a shift in thinking; endometriosis may not be just a gynaecological disorder, but indicative of a systemic problem.[64,65]

The cause of infertility is two-fold. Adhesion formation can result in distortion and loss of patency of the fallopian tubes. However, in women without adhesions fertility is still reduced; differences on a cellular level in eutopic endometrium are thought to be responsible. The American Society for Reproductive Medicine (ASRM) have developed a staging system to describe the extent of endometriosis and this correlates well with infertility (Table 1.1).

Table 1.1: ASRM criteria for endometriosis staging

Stage 1 (Minimal)	**Findings restricted to only superficial lesions and possibly a few filmy adhesions**
Stage II (Mild)	As above, some deep lesions are present in the cul-de-sac
Stage III (Moderate)	As above, plus presence of endometriomas on the ovary and more adhesions
Stage IV (Severe)	As above, plus large endometriomas, extensive adhesions

1.2.5: Endometriosis and Malignancy

Although endometriosis is a benign condition, it shares common characteristics with malignant cells.[66] Like cancer, endometriosis involves 'metastatic spread' attaching and damaging local and distant tissues.[67] A recent study, investigated differences in metastatic inducing proteins (MIPs) between eutopic endometrium from patients with endometriosis and those without. Immuno-histochemical and polymerase chain

reaction (PCR) analysis demonstrated S100A4, S100P, and AgR2 were expressed significantly more in endometriosis samples.[68]

Large studies also suggest endometriosis is associated with a 3-fold risk of endometroid and clear cell ovarian cancer.[69-70] Akin to its eutopic counterpart, ectopic endometrium can also exhibit epithelial abnormalities. In 2002, *Prefumo et al.* histologically reviewed ovarian endometriomas from 339 patients. Complex hyperplasia was significantly more prevalent in patients with ovarian cancer than in all other cases ($P<0.00001$), suggesting there is an association between ectopic complex hyperplasia and malignancy.[71] Despite endometriosis sharing certain traits with cancer, it is worth stressing ectopic tissue is *non-neoplastic*.

1.2.6: Medical management

Medical management encompasses analgesia and hormonal modulation. Often, medical management is empirical treatment, before the diagnosis has been confirmed. Some women wish to preserve fertility and avoid hormonal treatment, managing symptoms with complementary therapies or non-steroidal anti-inflammatories (NSAIDs). The effectiveness of NSAIDs remains inconclusive. Hormone modulation includes use of the combined oral contraceptive (COC), levenorgestrol intra-uterine system (LNG-IUS), or GnRH analogues. The COC is often the first line of hormonal management. It has been found to reduce pain scores significantly by 75%.[72] Danazol, a modified testosterone, is effective at treating endometriosis, although its androgenic side-effects make the drug undesirable and it has largely been replaced by GnRH analogues. The use of GnRH analogues induces a hypo-oestrogenic state. A systematic review of 1821 women taking GnRH

analogues showed a pain score improvement of 60-100%.[73] Unfortunately, GnRH analogues are restricted due to their side effects, as 6% of bone mineral density can be lost after only 6 months of therapy.[55]

A recent development is the use of aromatase inhibitors: aromatase is an enzyme, found in many tissues that catalyses the production of oestrogen. It is absent in eutopic endometrium from women without endometriosis, barely detectable in eutopic endometrium from women with endometriosis, and over expressed in ectopic lesions.[74] Unfortunately, these remain to be approved by the Food and Drug Association (FDA) for the treatment of endometriosis.

1.2.7: Surgical management

Ideal practice is to surgically remove ectopic lesions at the time of laparoscopic diagnosis. Preferably this would be the first intervention, as hormonal therapy should be stopped three months prior to diagnostic surgery in order to prevent under-diagnosis.[75] However, there is no general consensus in treating endometriosis; therapy very much remains individualised.

Surgical management involves lesion ablation, excision, adhelysis, neurectomies and with/without more radical surgery such as total abdominal hysterectomy (TAH) to prevent future lesions developing. Although ablative surgery is preferential to medical management, as ovarian function is not suppressed, some women still fail to respond – either due to incomplete excision or recurrence of the disease. In cases of severe pathology, and when fecundity does not wish to be preserved, a hysterectomy is indicated in addition to excision of lesions.

1.2.8: Aetiology

The aetiology of endometriosis is complex and is the subject of much debate. Several hypotheses are proposed, including retrograde menstruation (Sampson's theory), metaplastic change (Meyer's theory), embryonic rest cells (Cohnheim's theory) and lymphatic/haematogenous spread (Halban's theory). No single hypothesis can fully explain the disease spectrum, and pathogenesis is most likely to be multi-factorial.

1.2.8i: Sampson's theory

Originally proposed in 1927, Sampson's theory remains the most widely accepted. The concept relies on surgical observations made by Sampson and colleagues during surgery on women at the time of menses. Menstrual fragments were found to emanate from fallopian tubes; Sampson concluded that endometriosis was a consequence of retrograde menstruation.[76] However, Sampson was unable to demonstrate that shed fragments were capable of engraftment and survival outside of the uterus: this was later proven in a baboon model by *D'Hooge et al*.[77] The theory of retrograde menstruation is supported by other epidemiological data: women with Müllerian anomalies and cervical obstruction have an increased risk of developing the disease.[78]

The difficulty is that retrograde menstruation is a natural phenomenon, occurring in 90% of cycling women.[79] Why only 10% go on to develop endometriosis is unclear. It is unknown whether shed fragments are pathological to begin with, or whether it is exposure to a pathological pelvic cavity that facilitates adherence and growth. As it stands, it appears to be a mixture of the two. Therefore, although the theory of retrograde menstruation is attractive, it does oversimplify the disease. The theory is

deficient at explaining the presence of lesions at distant sites, such as the brain and lung, and in the non-menstruating such as post-menopausal women and men.[56,58,80,81]

1.2.8ii: Meyer's theory

Initially, it was assumed endometriosis only existed in the presence of peritoneum/mesothelia. Both peritoneum and endometrium share a common embryological ancestor – the coelomic cell. In 1924, *Meyer et al.* proposed coelomic epithelia were capable of differentiating into endometrium-like tissue in a process known as coelomic metaplasia.[82] The ability of coelomic epithelia to differentiate into 'endometrial glands' under the influence of oestrogen *in vitro* supports Meyer's hypothesis.[83] Scanning electron microscopy and histology have also revealed pelvic endometriosis is a *serial* change in ultra-structural characteristics from normal mesothelium.[84]

It is likely that metaplastic change is only responsible for 'subtle' lesions, which are visible microscopically. The theory is limited in its ability to explain why most cases occur in menstruating women, but can explain the 'exceptional cases of endometriosis' such as in men or distant sites. More research is needed to validate this theory, and it remains merely speculative.

1.2.8iii: Cohnheim's theory

In 1877, Julius Cohnheim first proposed the 'embryonic rest theory'.[85] Although the term was originally coined to describe carcinogenesis, it has subsequently been applied to understanding endometriosis. The theory is similar to Meyer's theorem, in that it relies on an embryological understanding of the reproductive tissue. However,

it has a clear distinction; lesions are not a result of peritoneal metaplasia, more they are a consequence of aberrant remnant embryonic cells. The Müllerian ducts are the embryological pre-cursors to the female reproductive organs (Section 1.1.5). The symmetrical and bilateral pattern of endometriotic lesions in the Pouch of Douglas has led some to speculate these may be cells of Müllerian origin, as they correlate with sites of duct migration.[86] It does explain the existence of lesions in men, but it cannot account for spread of disease to distant sites.

1.2.8iv: Halban's theory

Halban's theory hypothesises that endometriosis is the result of lymphatic or haematogenous spread of endometrial cells. Although appealing when trying to explain lesions at distant sites, it cannot elucidate why ectopic tissue is normally located in gravity-dependent locations.[87]

1.2.9: Endometriosis conclusion

Endometriosis is a highly prevalent debilitating disease, and management remains crude. Infertility is the side effect of most therapy, which is un-acceptable to a young and already sub-fertile cohort. A greater understanding of endometriosis pathogenesis is necessary to produce more sophisticated regimens.

1.3: Stem Cells

The term "stem cell" first appeared in scientific literature as early as 1868, coming from the German "Stammzelle" or "family cell". Ernst Haeckel, a phylogenist, used the term to describe a single cell, from which all multicellular organisms were believed to originate.[88] Although controversial at the time, the concept was later echoed by Russian histopathologist Alexander Maksimow and applied to the haematopoietic system in 1905.[89] It took another 50 years for theory to be replaced by fact, as a consequence of the pioneering work by *Till and McCulloch*.[90]

1.3.1: Terms and definitions

Stem cells (SCs) are defined as undifferentiated cells, capable of differentiating into other different cell types. They are mandatory for the development and maintenance of tissue.[91] Current dogma implies a cell needs to possess three qualities in order for it to be regarded as a SC.[92-94] These are:

- *Clonogenicity*

  This is the ability of single cells to form colonies.

- *Prolonged self-renewal and proliferative potential*

  This refers to the ability of a SC to undergo a high number of passages before cell death. Proliferative potential can also be determined by multiplying the total number of doublings of a single cell by serial passage until senescence.

- *Differentiation potential*

  Differentiation potential, synonymous with potency, is the ability of a SC to produce differentiated progeny. There is a scale as to how potent a SC is, and this essentially forms a 'stem cell hierarchy' (Figure 1.6) Over the course of

differentiation, SCs divide and gradually acquire markers and features of a differentiated state.

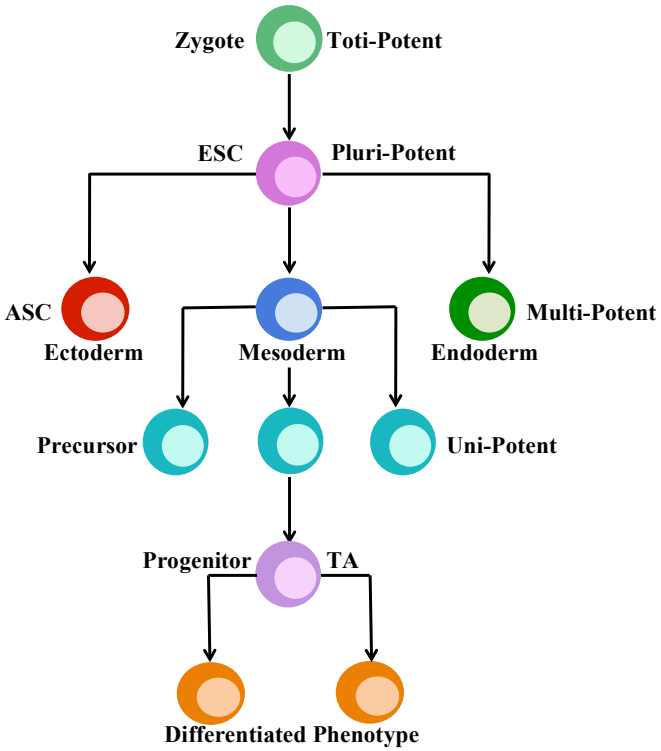

Figure 1.7 : Schematic diagram of 'Stem Cell Hierarchy'. The zygote is believed to be the most 'powerful' SC, capable of differentiating into all cell types. As division takes place, the potency of SCs becomes more restricted, until finally differentiating into a terminally differentiated phenotype.

The most potent of all mammalian SCs is the zygote; it has the ability to differentiate into embryonic and extra-embryonic tissue, and therefore is termed 'totipotent'. These differentiate to produce pluripotent SCs, which have the ability to form tissue from all three germ layers; the endoderm, mesoderm and ectoderm. Pluripotent SCs are found within in the inner cell mass of the blastocyst and are commonly referred to as embryonic stem cells (ESCs). As organogenesis ensues, SC potency becomes more restricted, producing multipotent SCs that give rise only to cell types within that germ layer; an example would be mesenchymal stem cells (MSCs). Cells that

differentiate into one cell type only are unipotent/precursors. A classic example of this is spermatogonia, which differentiate to produce the mature spermatozoa. Furthermore, there appears to be an even more restricted stem cell; one that is not even glorified with 'stem cell status'. Some lines of evidence suggests there exists a new population of cells, which are an intermediate between SCs and differentiated cells; these are referred to as transit amplifying cells (TAs). TAs, synonymous with progenitors, undergo rapid and repetitive cycles of cell divisions to increase in number. They acquire markers of the differentiated cell type, thus losing their ability for prolonged self-renewal.[95] The term adult stem/progenitor cell (ASPC) encompasses stem and progenitor cells in adult tissue.

1.3.1i: Cellular division

Cellular division is fundamental to understanding SC behaviour. SCs have the ability to undergo symmetric and asymmetric cellular division (Figure 1.7). Symmetric cell division maintains SC populations and thus facilitates self-renewal and clonogenic properties. Two identical daughter cells are produced, or in some cases progeny can be TAs. Asymmetric cell division facilitates differentiation. A SC will divide to produce an identical daughter cell along with a more differentiated TA cell. These TAs will rapidly divide to produce cells with a differentiated phenotype.

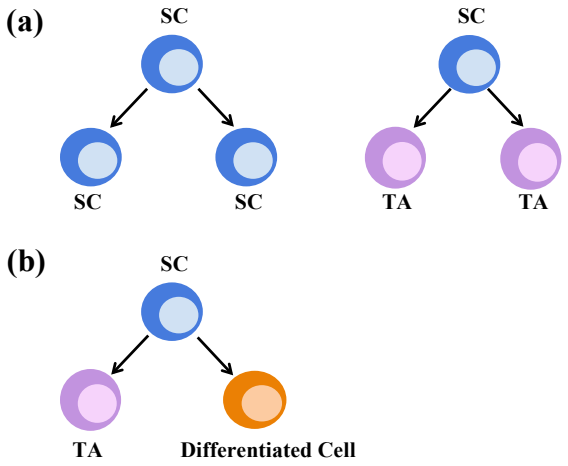

Figure 1.8: Schematic diagram of cellular division.
Symmetrical cell division **(a)** will give rise to identical SCs or TAs. Asymmetrical cell division **(b)** will result in a TA and a fully differentiated cell.

1.3.1ii: The stem cell niche

SCs reside in an anatomic structure called the niche. The niche can be regarded as a 'buffer environment', protecting SCs from differentiation, proliferation and apoptotic cues. Conversely, the niche is also responsible for stimulating ASCs to proliferate and differentiate at times of injury.[96] Niches have been identified and characterized in organs such as the skin and haematopoietic system.[97,98] The niche encompasses all surrounding components of the primitive SCs, including neighbouring mature cells, soluble components and the ECM. Each niche microenvironment and location is specific to its respective SC, and there is substantial evidence to suggest mesenchyme dictates epithelial differentiation.[99] Unfortunately, when taken out of its respective niche, SCs behave differently as to how they would *in vivo* and herein lies the main obstacle to SC research; efforts to study SCs *in vitro* remain futile. However, the advent of 3-dimensional (3D) culture is promising as evidence suggests that SCs cultured in a 3D environment behave similarly to how they would *in vivo*.[100] The most commercial 3D substrate is Matrigel^{TM}, a gelatinous extract

formed from Engelbreth-Holm-Swann mouse sarcoma cells. The full composition of the extract is unknown, causing much criticism. However it is rich in extracellular proteins such as laminin and collagen.[101] The application of 3D culture is often referred to as *ex vivo* as it is almost a hybrid of *in vitro* and *in vivo* research. Regardless of its difficulties, 3D culture has opened the door to understanding SC behaviour.

1.3.2: Embryonic stem cells

ESCs, first isolated from the mouse in 1981, are derived from the inner cell mass of the blastocyst.[102] ESCs are pluripotent and capable of differentiating into all 3 germ layers; mesoderm, ectoderm and endoderm. On account of their potency, ESCs show great promise for regenerative medicine. However, transplantation of ESCs *in vivo* has led to teratoma formation, and acquisition of ESCs provides an ethical headache.[102] There are attractive alternatives for autologous transplantation, in the form of induced pluripotent stem cells (iPS). The transcription factors *NANOG, OCT4* and *SOX2* are implicated to work synergistically at maintaining ESC phenotype.[103-105] The importance of these factors is exemplified by their forced expression in somatic cells to become iPS, and are therefore key markers of an undifferentiated state.[106]

1.3.3: Adult stem cells

ASCs, synonymous with somatic stem cells (SSCs), are quiescent undifferentiated cells, existing in small numbers in most organs after embryonic development.[107] They are fundamental units for tissue homeostasis, playing a critical part in the replenishment of dying cells, and regeneration of damaged tissue. With regards to

research and stem cell therapies, ASCs are a more attractive alternative to ESCs as acquisition is less controversial. Similarly, *in vivo* transplantation does not pose the risk of teratoma formation. However, ASC research is not without its difficulties and these shall be discussed shortly.

1.3.3i: Plasticity

Between 2000 and 2001, four high profile papers were published suggesting ASC plasticity.[108-111] It was proposed ASCs can trans-differentiate (acquire broader developmental potential), an exciting prospect that heralded enthusiasm for ASC therapy. Prior to this, ASCs were believed to be restricted to cell lineages within the germ layer they originate, i.e. multipotent only. *Clarke et al.* labelled neural ASCs (ectodermal derived) with β-galactosidase and co-cultured with embryoid bodies. Surprisingly, β-galactosidase positive cells were also positive for desmin and myosin heavy chain, both markers of myocytes (mesoderm derived). However, two articles both published in *Nature* the following year raised doubt as to whether trans-differentiation did actually occur. *Ying et al.* and *Tereda et al.* aimed to produce embryonic-like SCs from co-culture of ESCs with neural ASCs/haematopoietic ASCs *in vitro*.[112,113] Resulting embryonic-like SCs had twice the normal DNA content, suggesting that fusion had in fact taken place, and there has been a subsequent endeavour to repeat the findings of *Clarke et al.* and others. More recently, evidence suggests that a small group of pluripotent stem cells may reside in adult tissue in a dormant state. These cells are referred to as 'very small embryonic like' stem cells (VSEL-SCs) and display pluripotency *in vitro*.[114] It could be these cells that are responsible for the results of *Clarke et al*. As it stands, the jury is still

out as to whether trans-differentiation of ASCs is possible, and plasticity remains a source of contention amongst stem cell biologists.

1.3.3ii: Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent adult progenitor cells, which have the ability to differentiate into multiple mesenchymal lineages, including adipocytes, osteoblasts and chondrocytes.[115] They can be derived from numerous sites, most commonly bone marrow, adipose tissue and umbilical cord blood. Despite efforts, specific MSC markers are lacking due to poor specificity. Currently, combined expression of Stro-1 and CD106 is considered a good candidate MSC marker.[116] As it stands, MSCs remain the 'gold standard' multipotent ASC, and the subject of much research by both scientists and clinicians alike.

1.3.3iii: Stem cell markers

As ASPCs exist in low numbers in mammalian tissue, definitive markers are required to identify and enrich cell populations for further study. This poses a real difficulty: markers have been identified for some ASPCs, but none are without their limitations. The most reliable markers of an undifferentiated state are those of NANOG, OCT4 and SOX2. Unfortunately, these are transcription factors and located within the cell nucleus; isolation of viable cells is only possible via magnetic cell sorting (MACS) or fluorescence-activated cell sorting (FACS) if markers are on the cellular surface. Discovery of a specific ASPC surface marker is an active area of research. The difficulty is specificity, for example; CD34 is a marker of hematopoietic APSCs but it is also present on mature endothelial cells.[117] A cell that expresses a stem cell marker may not be able to function like a SC, for example,

Stro-1+ cells, a putative MSC marker, were unable to demonstrate clonogenicity in the endometrium.[118] For this reason, SCs are often defined by the presence of markers associated with that specific lineage but the absence of markers of a more differentiated state. Therefore in order to validate an ASPC marker, *in vitro* and *in vivo* assays are mandatory.

1.3.4: Stem cell research

As ASPC markers are unreliable when used in isolation, multiple assays are required for validation by characterizing 'stem cell' function. Each of these assays is not without limitations.

1.3.4i: In vitro assays

These require an assessment of the defining features of SCs (Section 1.3.1). Clonogenicity calculates the ability of putative SCs, when seeded at very low densities, to produce colonies of daughter cells. Prolonged self-renewal requires serial passaging of cells before cell death and apoptosis. *In vitro* differentiation is performed by culturing cells in media supplemented with various growth factors, implicated at inducing differentiation into a specific cell type. Histochemical staining along with gene expression confirms differentiation.

1.3.4ii: In vivo assays

The side population phenotype (SP) could be classified in either of the *in vitro* or *in vivo* camps. The assay requires FACS to isolate a population of cells enriched with SCs, and was first described over 15 years ago when studying bone marrow.[119] Hoescht 33342 is a fluorescent dye that binds to the minor groove of

deoxyribonucleic acid (DNA). Uptake of the dye is universal and occurs in all cells. However, to be extruded, the process is metabolic and requires a sufficient amount of ATP-binding cassette (ABC) transporters. If SCs had DNA damage, this would mean all progeny would be affected and it is therefore believed SCs have the ability to extrude Hoescht dye as a safety mechanism. The dye emits fluorescence when excited by UV light, enabling detection and sorting via FACS. The cells that do not possess the fluorescent activity i.e. the cells that have extruded the dye are the side population (SP). Cells are counter stained with propodium iodide (PI) to ensure viability. There are limitations with the SP assay; Hoescht can be toxic and the effect of SP on cell survival can be poor.[120] There is also growing caution that dye efflux ability is not ubiquitous nor unique to all SCs.[121,122]

In 1975, John Cairns proposed the immortal DNA strand hypothesis: when SCs undergo mitosis, DNA is divided asymmetrically.[123] The parent strand is retained by SCs and the new strand is selectively segregated to a daughter cell. This would mean spontaneous mutations only occur in the newly synthesised strand, preventing genetic errors in SCs and potential cancer risk. Although controversial, the principle forms the basis for the label retaining cell technique (LRC). Parental DNA is marked with a DNA label during S phase of the cycle, typically bromodeoxyuridine (BrdU). If cells undergo symmetric mitotic division the DNA label would rapidly be chased out of the cell. If however, the cell undergoes asymmetric DNA division, the BrdU will segregate in the SC and will still be detectable after a number of divisions. Antibodies specific to BrdU can then be used to identify LRCs in tissue. The main drawback is that BrdU binds to DNA during replication instead of thymidine.

Consequently, mutations occur and BrdU is a recognized health hazard; use of the LRC technique in humans is not permitted.

The 'gold standard' of *in vivo* assays is tissue reconstitution. This comprises a series of experiments whereby the tissue of interest is reconstituted in a recipient animal by transplantation of donor cells.[96] Most commonly, bone marrow derived stem cells (BMDSCs) are injected intra-venously (i.v.) into immune compromised mice. Alternatively, they can be transplanted subcutaneously (s.c.) or under the kidney capsule. Animals are later sacrificed and explants examined. Although informative, experiments are limited by the absence of a native stem cell niche and the inability of the explant to develop into a functional organ.[96]

1.3.5: Stem cell conclusion

Despite being a rapidly evolving and exciting field, stem cell research remains in its infancy. Due to ethical restrictions, most work focuses on ASPCs. However, study of these SCs is not without its own set of difficulties, and no paradigm can be applied to explain the behaviour of ASPCs in all tissues. Definitions and theories persist, yet each is open to interpretation. The lack of a definitive ASPC marker requires the use of *in vitro* and *in vivo* assays to characterise putative ASPC phenotype.

1.4: Endometrial Stem Cells

Nearly all somatic organs contain populations of ASPCs. ASPCs were first hypothesised to exist in the haematopoietic system, as erythrocytes have a fixed lifespan of only 90 days.[89] If we compare the haematopoietic system with the endometrium; we know human endometrium has an incessant and rapid cycle of proliferation every *28 days*. Therefore, it would be logical to assume an ASPC is also responsible for the maintenance of this tissue.

1.4.1: Early work

Prianishnikov was the first to consider endometrial ASPCs. In 1978, he proposed endometrial ASPCs reside in the deep basal layer, and their differentiation is marked by changes in hormonal receptivity.[124] *Prianishnikov* suggested hormone independent ASPCs initially differentiate into oestrogen dependent cells, then on to become both oestrogen and progesterone dependent. Terminally differentiated cells were progesterone dependent only, and had a limited lifespan (Figure 1.8).

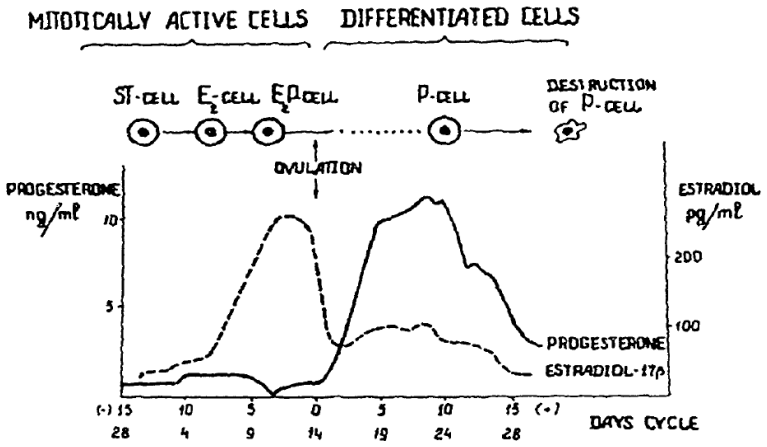

Figure 1.9: Hypothesised hormonal receptivity of endometrial ASPC. Endometrial ASPCs are believed to be devoid of oestrogen and progesterone receptors, acquiring such receptors during differentiation. [124]

The theory was not supported until 1991. *Padykula et al.* were able to demonstrate zonal differences in proliferative ability in the endometrium; zones I and II had high mitotic activity, whereas zones III and IV had reduced activity, suggesting the existence of a group of quiescent ASPCs in the basal layer (Figure 1.9).[125] Similar results were observed by *Brenner et al.,* using the more specific proliferative marker phospho-histone-H_3 (PPH_3), in macaque endometrium.[126]

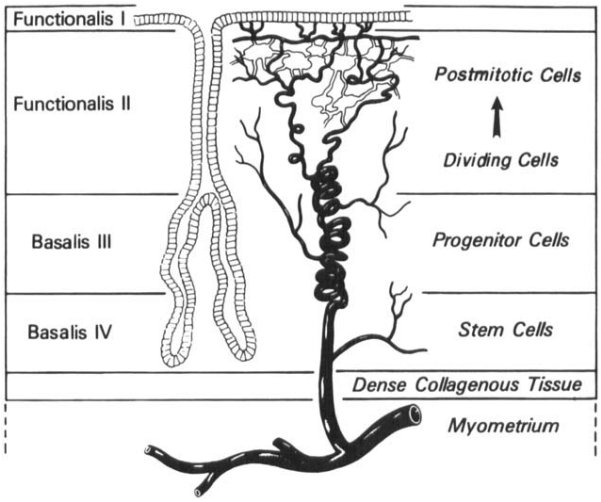

Figure 1.10: Hypothesised location of ASPC in human endometrium.[125]
Schematic diagram demonstrating the hypothesised location of endometrial ASPCs. Reduced mitotic activity in the basalis layer (zones III and IV) is consistent with stem cell activity.

There certainly is indirect evidence to support the combined theory of *Prianishnikov* and *Padykula et al.* Electron microscopy has revealed endometrium is able to expand between 4-7mm within 4-10 days, and growth is initiated from the necks of 'de-nuded' glands in the basalis.[36,127]

Also, a Japanese PCR study investigated the monoclonal composition of endometrial tissue.[128] The authors examined CAG tags on the inactivated X chromosome of endometrial epithelia. It was discovered glands within a $1mm^2$ area were 60% likely to be monoclonal, suggesting the presence of a single progenitor cell.

1.4.2: Clinical evidence

Electro-surgical ablation aims to remove all endometrium from the uterine cavity. Nonetheless, complete re-growth has been found as little as 4 months after therapy.[129] Furthermore, post-menopausal (PM) endometrium loses the functional layer and only the basalis remains. However, if given sufficient exogenous hormones, it is capable of regenerating a new differentiated functionalis that can support a viable pregnancy to term.[130] Similarly, Asherman's syndrome is defined as loss of endometrium and patency of the uterine cavity, secondary to adhesions. Under the influence of oestrogen, injection of autologous bone marrow cells into the uterine cavity has been reported to stimulate endometrial re-growth, sufficient to support *in vitro* fertilisation (IVF) pregnancy.[131]

In addition; bone, cartilage, smooth and striated muscle have all been observed in human endometrium, mainly in stromal sarcomas.[132-134] The endometrium can also be a source of extra-medullary haematopoiesis, albeit in rare circumstances.[135] A defining feature of MSCs is their ability to differentiate into mesodermal tissue when given external prompts.[115]All findings considered, there could be a multipotent SC in the endometrium that has the ability to generate erroneous tissue, if given pathological stimuli.

1.4.3: Endometrial adult stem/progenitor cell assays

1.4.3i: In vitro assays

Despite early data suggesting ASPC existence, conclusive evidence did not emerge until 2004. *Chan et al.* demonstrated clonogenicity of endometrial derived cells, definitive of a SC phenotype.[136] Endometrial epithelial and stromal cells were seeded

at clonal density, and cloning efficiency (CE) was assessed after 15 days in culture. Two types of colonies developed; large tight packed ones, consisting of over 4000 cells, and more common smaller dense colonies. CE of epithelia was 0.22 ± 0.07%. Stroma had an overall CE of 1.25 ± 0.18%.[136] Large colonies could also undergo 30-32 population doublings before cell senescence.[137] The authors hypothesised that smaller colonies represented TAs as they were more common and had limited self-renewal. Larger colonies were believed to represent primitive ASCs; these were less common and more proliferative. Other authors have produced similar results in stroma, from normal and pathological endometrium.[138-140] Serial passage of putative epithelial and stromal ASPCs before senescence is reported as 12 and 15-30 respectively.[139,141]

The idea that the endometrium, a somewhat disposable tissue, could be a source of other cell types has captured the imagination of stem cell biologists. As mentioned previously, endometrium derives from intermediate mesoderm (Section 1.1.5). Therefore, if multipotent it should be able to produce other mesoderm cells types such as adipocytes, osteoblasts, chondrocytes and skeletal muscle. A number of studies suggest endometrial stromal adult stem/progenitor cells (ESt-ASPCs) from intact endometrium are capable of multi-lineage differentiation *in vitro*, being able to differentiate into fat, bone, cartilage, and skeletal muscle. [138, 141-149] In addition, ESt-ASPCs have demonstrated plasticity by trans-differentiation into neural and pancreatic cells both *in vitro* and *in vivo*.[146,150,151]

Meng et al. were the first to publish similar results using ESt-ASPCs obtained from menstrual blood.[152] Cells were capable of differentiation into adipocytes, osteoblasts,

chondrocytes, cardiocytes, myocytes and endothelia. ESt-ASPCs from menstrual blood were also capable of trans-differentiation into endodermal and ectodermal tissue such as hepatocytes, pulmonary epithelia and neurones. ESt-ASPCs in question were mononuclear and demonstrated positive immuno-reactivity for CD90, CD73 and CD103, but were devoid of CD34 and CD45 suggesting a mesenchymal, not haematopoietic origin. Others have demonstrated similar features of ESt-APSCs derived from menstrual blood, and this has sparked a campaign of interest in ESt-ASPCs as a source of SC therapy.[153-159] Animal models of Duchenne muscular disease, stroke, diabetes and critical limb ischemia suggest ESt-ASPCs improve outcome.[150,151,154,159] In one phase I clinical trial of 4 patients with multiple sclerosis, no adverse effects have so far been documented.[160] Similarly, a phase II double-blind clinical trial using menstrual ESt-APSCs for the treatment of congestive heart failure is underway, and another for the treatment of critical limb ischemia has just been launched.[161,162] Interesting, the use of ESt-ASPCs for endometrial diseases, such as Asherman's syndrome, is less heavily researched.

Most work assessing multipotency of endometrial cells has focused largely on the stromal population, probably on account the greater abundance in endometrial tissue, and the associated difficulties with epithelial culture. To the best of our knowledge, only three studies have attempted to differentiate primary endometrial epithelial adult stem progenitor cells (EEp-ASPCs). In 2009, *Gargett et al.* were able to demonstrate unipotency, by differentiating EEp-ASPCs into CK+ gland-like structures, in 3D Matrigel™.[143] Unfortunately, an assessment of multipotency was not possible, as epithelial cells were unable to grow to confluency. This may be because authors were using colony-forming units from EpCAM+ endometrial epithelia, a notoriously small

subpopulation of cells. Two papers by *Cervello et al.* suggested that endometrial epithelial SP are multipotent, by demonstrating differentiation of these cell types into adipocytes and osteoblasts *in vitro*.[141,147] Cells were passaged up to 15 times and cultured in appropriate induction/non-induction media. After 2 weeks in culture, EEp-ASPCs in adipogenic induction media demonstrated the presence of Oil Red O lipid vacuoles, unlike cells in control media. Likewise, EEp-ASPC cultures in osteogenic induction media demonstrated positive immuno-reactivity for bone sialoprotein (BSP) when compared to non-induced cells. Published figures are open to criticism, as despite the presence of lipid vacuoles, differentiated EEp-ASPCs are spindle-like and do not display the characteristic rounded morphology typical of adipocytes (Figure 1.10). Similarly, PCR was semi-quantitative and therefore comparisons cannot easily be made. Curiously, the same authors were able to demonstrate unipotency of endometrial SP cells *in vitro*.[141] EEp-ASPCs and ESt-APSCs were injected under the mouse kidney capsule (mesodermal derivative) and generated endometrial tissue; cells did not differentiate into kidney parenchyma. Therefore, an assessment of the differentiation potential of endometrial epithelia still remains elusive, tenuous and lacking.

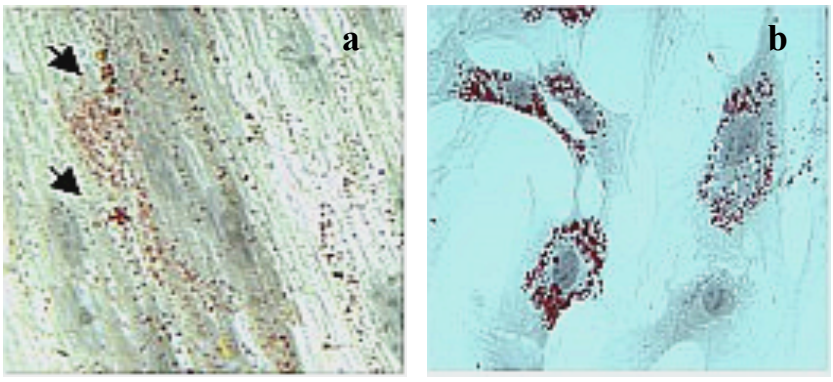

Figure 1.11: Adipogenesis of human endometrial epithelial SP cells[141]
Epithelial SP cells cultured in adipogenic media displayed Oil Red O Lipid vacuoles **(a)**, see arrow. However, morphology was dissimilar to control adipocytes **(b)**.

1.4.3ii In vivo assays

SP cells have been isolated in epithelia and stromal fractions from healthy and cancerous endometrium.[141,147,163-9] Higher numbers have been reported in menstrual and proliferative phases, consistent with endometrial ASPC function.[163,164] A mouse model was able to confirm that stromal SP cells significantly increased 6 hours after endometrial injury, and was dependent upon the presence of oestrogen.[168] There does not appear to be any change in SP numbers throughout a woman's life, again consistent with the dormant nature of ASPCs.[147] Studies have also assessed the clonogenicity, proliferation and differentiation potential of these cells *in vitro* and results appear to be consistent with SC theory.[141,147,163,164,166,167] Tissue reconstitution *in vitro* has proven more difficult at regenerating endometrial tissue.[141,147] However, tissue reconstitution has been successful at generating endothelial cells and mature blood vessels in the mouse kidney.[167] Microarray studies show up-regulation of 44 genes and down-regulation of 14 in SP cells when compared to non-SP, suggesting a common cell fate.[147] However, there is also evidence to suggest SP cells are heterogeneous. Reduced expression of epithelial and stromal markers CD9 and CD13 have caused some authors to claim that SP cells are representative of cells in an undifferentiated state.[163] However, others have cast aspersions on this observation, and question whether in fact a large proportion of SP cells are blood borne. Immuno-staining with the SP marker, ABCG2+ has located these cells in basal epithelia and as CD31+ endothelial cells throughout the endometrium.[164,167]

Three papers have demonstrated the presence of LRCs in endometrium. The first, by *Chan et al.*, pulse labelled mouse endometrium with BrdU.[170] After an 8 week chase, only 3% of epithelial nuclei were $BrdU^{+}$. These were in the luminal epithelia and

dual labelling immuno-fluorescence revealed cells were negative for ER-α. Conversely, 6% of stroma were LRCs; these were located just below the luminal epithelia, at the endo-myometrial junction or near blood vessels. Interestingly 16.7 ± 0.02% of these cells were ER-α negative. Co-labelling with CD45 and CD31 revealed these cells were not leucocytes nor endothelia. $BrdU^+$ cells surrounding blood vessels were positive for α-SMA and it is probable these cells represent pericytes. The same authors demonstrated oestrogen drives epithelial LRC proliferation in juvenile development, but has a minimal role in epithelial and stromal LRC cyclical regeneration, suggesting that neighbouring $BrdU^-/ER\text{-}\alpha^+$ cells release paracrine factors to mediate an LRC response.[171] *Cervello et al*. also had difficulties in detecting epithelial LRCs after a 3 week chase. Stromal LRCs were detected in similar numbers and 0.2 ± 0.1% of stromal LRCs co-expressed OCT4, a marker of an undifferentiated state.[172] All considered, as $BrdU^+$ epithelia were lost soon into the chase period, it could be that epithelial regeneration relies on the self-duplication of a mature epithelial cell type. Conversely, it may be that the LRC technique is not sensitive enough to label endometrial epithelial cells with an ASPC phenotype.

In addition, as BrdU is toxic, the subject for these assays is mouse endometrium. Mice do not undergo a menstrual cycle; they undergo an estrous one. The cycle is still dependent upon the female sex hormones oestrogen and progesterone, however the cycle is shorter, typically 4-5 days and endometrial tissue is not shed: it is reabsorbed. Although some authors champion the mice as a sufficient model to study endometrial ASPC behaviour, one cannot be certain it is applicable to understanding ASPCs in *human* endometrium.

As touched on previously, tissue reconstitution has been attempted *in vivo* by animal xenograft-transplants.[141,147,167] Results have demonstrated an ability to produce endometrial tissue but explant survival is poor. *Cervello et al.* also investigated the relationship between stromal and epithelial ASPCs.[147] When stromal SP cells were transplanted under the kidney capsule in mice, only endometrial stroma was formed. A similar observation was observed when only epithelial SP cells were transplanted. Endometrial-like tissue was only generated when both these populations were combined. This would suggest that two distinct ASPCs exist; a stromal and an epithelial ASPC.

1.4.4: The endometrial stem cell niche

It would be sensible to assume that the endometrial stem cell niche is confined within the basal layer of the endometrium; this is the only layer that persists throughout all stages of the menstrual cycle and menopause. Indeed, there is evidence to suggest the clonogenicity of putative endometrial stem cells remains constant throughout all stages of the menstrual cycle and in the post-menopausal.[173] In addition, ABCG2+ cells, representative of the SP, have been found in basal epithelia.[164] However, the same authors found ABCG2+ cells in stroma throughout the endometrium. This finding is supported by the fact cells with SC activity are present in menstrual blood, indicating ASPCs may also reside in the functional layer.[152] Cells positive for the marker of an undifferentiated state, SOX2 were located in peri-vascular stroma.[174] Likewise, basal stroma were also found to be positive for haematopoietic ASPC maker c-kit.[175] Location of the stem cell niche in other tissues has relied on the LRC phenotype. As previously discussed, in the case of endometrium, results are not necessarily applicable to human physiology. Epithelial LRCs are located at the

luminal epithelia and it is likely, as mice do not have a basal or functional layer, that this is the source of endometrial regeneration.[170] Stromal LRCs were observed in three groups; around blood vessels, at the endo-myometrial junction and also under the luminal epithelia. It could be possible that there exists more than one niche for each APSC; it could also be possible that each of these LRCs represent a different APSC population, each with its own respective niche.

As mentioned in Section 1.3.1, the niche senses the need for tissue replacement. One could be forgiven in thinking that stimulatory signals involve changing concentrations of oestrogen and progesterone. However, this is not necessarily the case. BrdU+ epithelial cells were negative for ER-α, whilst surrounding epithelia were positive. Similarly, only 15.7% of stromal LRCs had positive immuno-reactivity for ER-α and yet both cells were able to proliferate under the influence of oestrogen.[170] *Schuring et al.* substantiate these findings, as expression of ER-α and ER-β is believed to be down-regulated in clonal stem cell cultures.[176] Therefore, endometrial ASPCs may not be subject to direct stimulation of the sex steroids, but rather depend on paracrine mechanisms within the stem cell niche.

1.4.5: Bone marrow as a source of endometrial ASPCs

Recent studies suggest the bone marrow (BM) may be responsible for endometrial regeneration. Bone marrow derived cells (BMDCs) have been shown to circulate and contribute to repopulating neurons, hepatocytes, and cardiomyocytes.[177-179]

In 2004, *Taylor et al.* was able to demonstrate chimerism in endometrial glands and stroma of four women who received human leukocyte antigen (HLA) mismatched

bone marrow transplants.[180] Frequency of chimerism in $CD45^-$ cells ranged from 0.2% to 52% and there was an association with time from transplant. Whole glands and neighbouring stroma were of donor origin, suggesting clonal expansion of a common ancestor. On account of the limited numbers, it could not be determined whether this was significant. The study was also flawed in that the results were observed in a pathological model; endometrium and bone marrow from recruited patients were both pathological.

BM as a source of endometrial regeneration is supported by the ability of BM derived MSC to produce 'decidua-like' stroma after activation of the PKA pathway *in vitro*.[181] Similarly, BM derived cells have been found in the decidua of normal murine pregnancy.[182] Co-culture of BMDC with endometrial stromal cells and oestrogen stimulation resulted in CK^+ endometrial epithelial like cells.[183] However, this study is controversial as it is questionable as to how efficient the filtration process, aimed at preventing epithelia contamination, actually was. A more elegant transgenic study allowed green fluorescent protein (GFP) labelling of donor CD45 cells.[184] These were transplanted into wild mice, and endometrial luminal epithelia were found to be GFP+. Unexpectedly, GFP+ endometrial cells rose dramatically to 82% in one pregnant mouse. These findings, together with the knowledge that LRCs and SP cells are located peri-vascularly, support that BM could be a potential source of endometrial regeneration. However, it is questionable as to whether BM is the sole source of endometrial regeneration or merely a replenishing ally. In one study, male BMDCs were transplanted i.v. into female mice. Fluorescence in situ hybridization (FISH) analysis of mice endometrium, found Y-chromosomes were present in only 0.0002% of CD45-/F4/80- epithelial cells and 0.0003% of CD45-/F4/80- stromal

cells.[185] Likewise, a Spanish study of women who received BM transplant from male donors, revealed Y-chromosomes were found in the non-SP population.[186] There is conclusive evidence to suggest BM is a source of endometrial regeneration but contribution is low. It is likely BM is implicated in endometrial repair after times of injury such as ablation, and in the formation of the decidua, when the endometrium requires 'extra-assistance'; a resident ASPC is most likely to be responsible for cyclical restoration.

1.4.6: Stem cells in endometriosis

Although stem cells are essential for tissue replenishment, they can have a sinister alter ego; cancer stem cells are known to exist in a number of tissues. Since endometriosis shares many common features with malignancy, it is possible that 'endometriosis stem cells' also co-exist. The implication of stem cells in endometriosis does not refute nor object any pre-existing concept. In fact, it can be applied to, and work synergistically with each hypothesis in turn.

In the case of Sampson's theory, retrograde menstruation of *stem cells* could be responsible. This would explain why lesions are not 'lost' with each menstruation. It also justifies lesion proliferation and high recurrence rates. Endometrial stem cells are hypothesised to exist in the basal layer. We know that women with endometriosis shed more of their basal endometrium than healthy controls.[187] However, there is one weakness with this collaboration of theories: if endometrial stem cells were thrust into the pelvic cavity, they would inevitably be taken out of their respective niche and presumably into the niche of another organ. If this were so, it is unknown why ASPCs would not differentiate into tissue from that respective niche, for example

ovary or peritoneum. It could be that healthy endometrial stem cells are unipotent. It could also be that retrograde menstruation involves the expulsion of multipotent ASPCs in the majority of women. These could contribute to surrounding tissue, going unnoticed; but in the 10% that do develop endometriosis, these could be 'pathologically committed,' and it is this commitment step that is integral to pathogenesis. We know that ESt-ASPCs are multipotent in cases of health and endometriosis, but the same cannot be said for EEp-ASPCs.

Likewise, the stem cell theory also supports that of Meyer, Halban and Cohnheim. With respect to Meyer's theory, bone marrow is a source of endometrial regeneration and is also recruited in endometriotic lesions; coelomic metaplasia could be from these cells.[185] Furthermore, smoking inhibits bone marrow recruitment and appears to have a protective effect upon endometriosis.[188,189] The aetiology of endometriosis is controversial and theories remain plentiful; nonetheless the concept of pathological stem cells is applicable to all.

1.4.7: Putative Endometrial APSC Makers

Co-expression of CD146 and platelet derived growth factor–receptor β (PDGF-Rβ) is currently being championed as an ESt-ASPC marker.[149] FACS sorted $CD146^{+}$/PDGF-$R\beta^{+}$ cells had significantly greater colony-forming capacities than $CD146^{-}$/PDGF-$R\beta^{-}$ cell populations, 7.7 ± 1.7% verses 0.7 ± 0.2% respectively (p=0.0001).[149] Similarly, $CD146^{+}$/ PDGF-$R\beta^{+}$ cells produced more large colonies with densely packed cells and a high nuclear:cytoplasmic ratio, thought to be representative features of ASCs. More lately, W5C5 has also been heralded as a putative ESt-ASPC marker.[190] W5C5+ cells represent 4.2 ± 0.6% of endometrial

stromal cells, which are located peri-vascularly and have greater clonogenicity than depleted counterparts.

Until very recently, efforts to describe an EEp-ASPC marker remained forthcoming. Data from our laboratory suggest stage specific embryonic antigen -1 (SSEA-1) may be a potential candidate. SSEA-1 is a cell surface glycan and present on mouse ESCs. Expression is lost during ESC differentiation, and therefore its presence signifies cells in an undifferentiated state.[191] Expression is not unique to ESCs, as it has also been found to be an ASPC marker in the human nervous system.[192]

Screening endometrial full thickness samples throughout the menstrual cycle demonstrated that SSEA-1 immuno-reactivity was specific to epithelia only. In addition, staining intensity was significantly greater in proliferative endometrium than in secretory (unpublished data). Conventional ASPC assays have demonstrated SSEA-1 enriched cell populations have a greater propensity to produce gland-like structures in 3D culture, when compared to SSEA-1 depleted fractions (Figure 1.11). However, work on SSEA-1 phenotype remains preliminary and ASPC assays are required to validate SSEA-1 as a suitable EEp-ASPC marker.

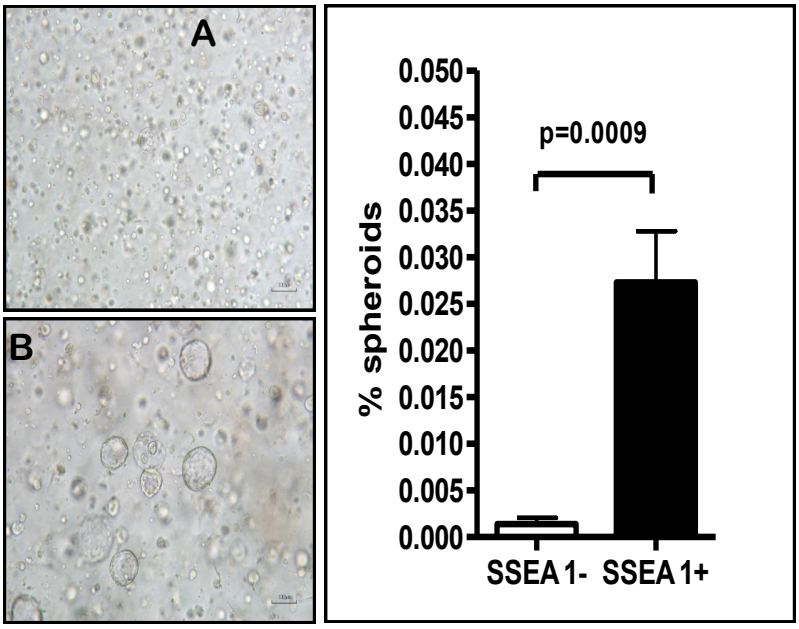

Figure 1.12: Spheroid formation of SSEA-1 enriched and depleted cell populations. SSEA-1 enriched **(b)** and depleted **(a)** cell populations were cultured in 3D media to produce gland-like structures. SSEA-1 enriched populations had greater propensity to produced gland-like structures/organoids (unpublished data).

1.4.8: Endometrial Stem Cell Conclusion

Indirect evidence suggests that the endometrium is home to a group of ASPCs, and this has been supported by functional assays. *In vitro* studies have demonstrated a group of epithelial and stromal cells with high clonogenicity, multipotency and prolonged self-renewal. SP cells, LRCs and markers of differentiation are all present within the endometrium. However tissue reconstitution of endometrium *in vivo* remains disappointing. A greater understanding is required, but is dependent upon the acquisition of endometrial ASPC markers. Co-expression of CD146/PDGF-Rβ and W5C5 are representative of putative ESt-ASPC markers. ESt-ASPCs are unable to generate endometrial glands and tissue as a whole, suggesting the existence of a distinct EEp-ASPC. Recently, SSEA-1 was proporsed as a potential EEp-ASPC marker. In order to validate this hypothesis, ASPC assays need to be employed, comparing epithelial SSEA-1+ and SSEA-1- fractions in the endometrium.

Chapter Two:

RESEARCH AIMS

Recently, SSEA-1 has been proposed as a putative stem cell marker in endometrial epithelia. This work aims to validate the stem cell behaviour of SSEA-1 human endometrial cells, by specifically looking at its differentiation potential.

2.1 Assessment of unipotency in vitro

SSEA-1 enriched cells have a greater propensity to produce 'gland-like structures' in 3D culture. A thorough immuno-histochemical analysis comparing *in vivo* endometrial glands from pre- and post-menopausal tissue, with gland-like structures generated from 3D culture will be conducted. This will establish whether the structures really are representative of endometrial glands. The results therefore will confirm, for the first time, an endometrial epithelial cell subpopulation with progenitor function.

2.2 Assessment of multipotency in vitro

Mesenchymal stem cells are capable of differentiating into different cell types of mesodermal origin. Previous authors have shown that human ESt-ASPCs have the capacity to differentiate into these cell types. This study aims to establish whether SSEA-1 enriched and depleted epithelial cells have the same potency.

2.3 Assessment of pluripotency in vitro

Eutopic and ectopic endometrium from women with endometriosis contains increased neural fibres when compared to normal controls. The origin of these nerve fibres is unknown. It is possible they are the result of aberrant differentiation of a pluripotent endometrial stem cell. ESt-ASPCs can trans-differentiate to produce cell types from endodermal and ectodermal germ layers. This work will also investigate whether SSEA-1 enriched and depleted epithelial cells possess the same plasticity.

Chapter Three: METHODS

3.1: Overview of Study Design

3.1.1: Unipotency experiments

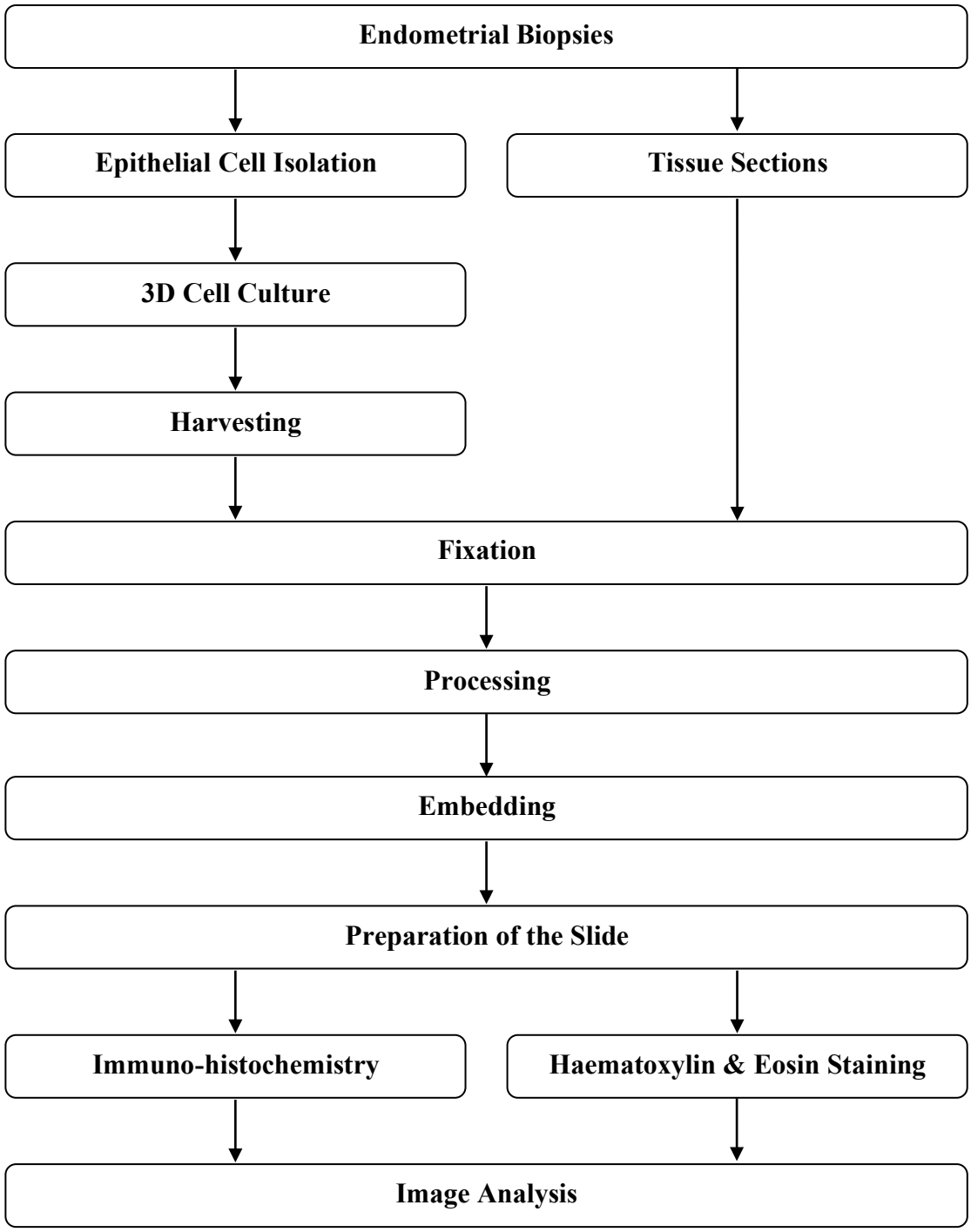

Figure 3.1: Flow diagram of study design to assess unipotency of SSEA-1 enriched cell populations

3.1.2: Multipotency experiments

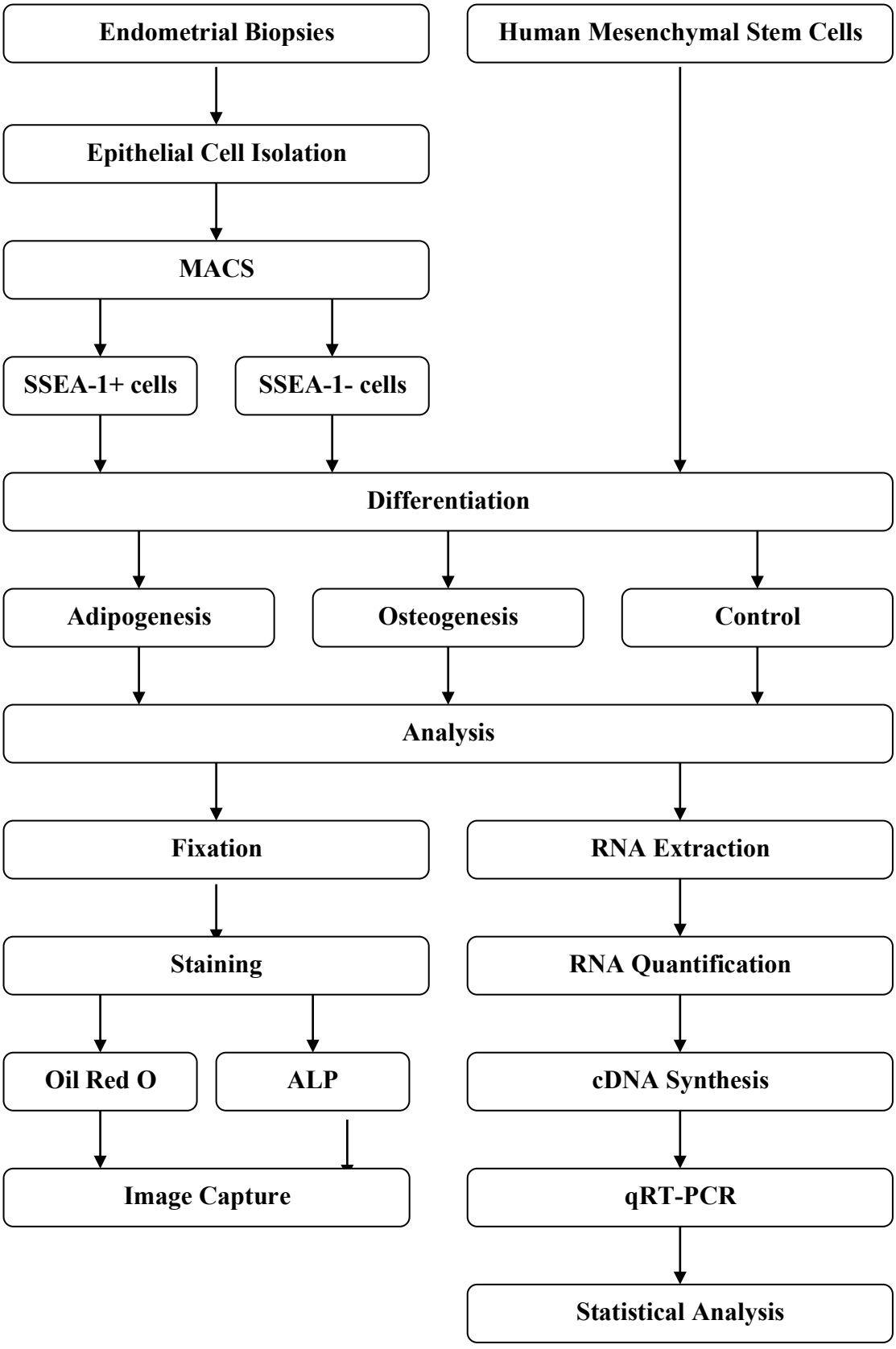

Figure 3.2: Flow diagram of study design to assess multipotency of SSEA-1 enriched and depleted cell populations

3.1.3: Pluripotency experiments

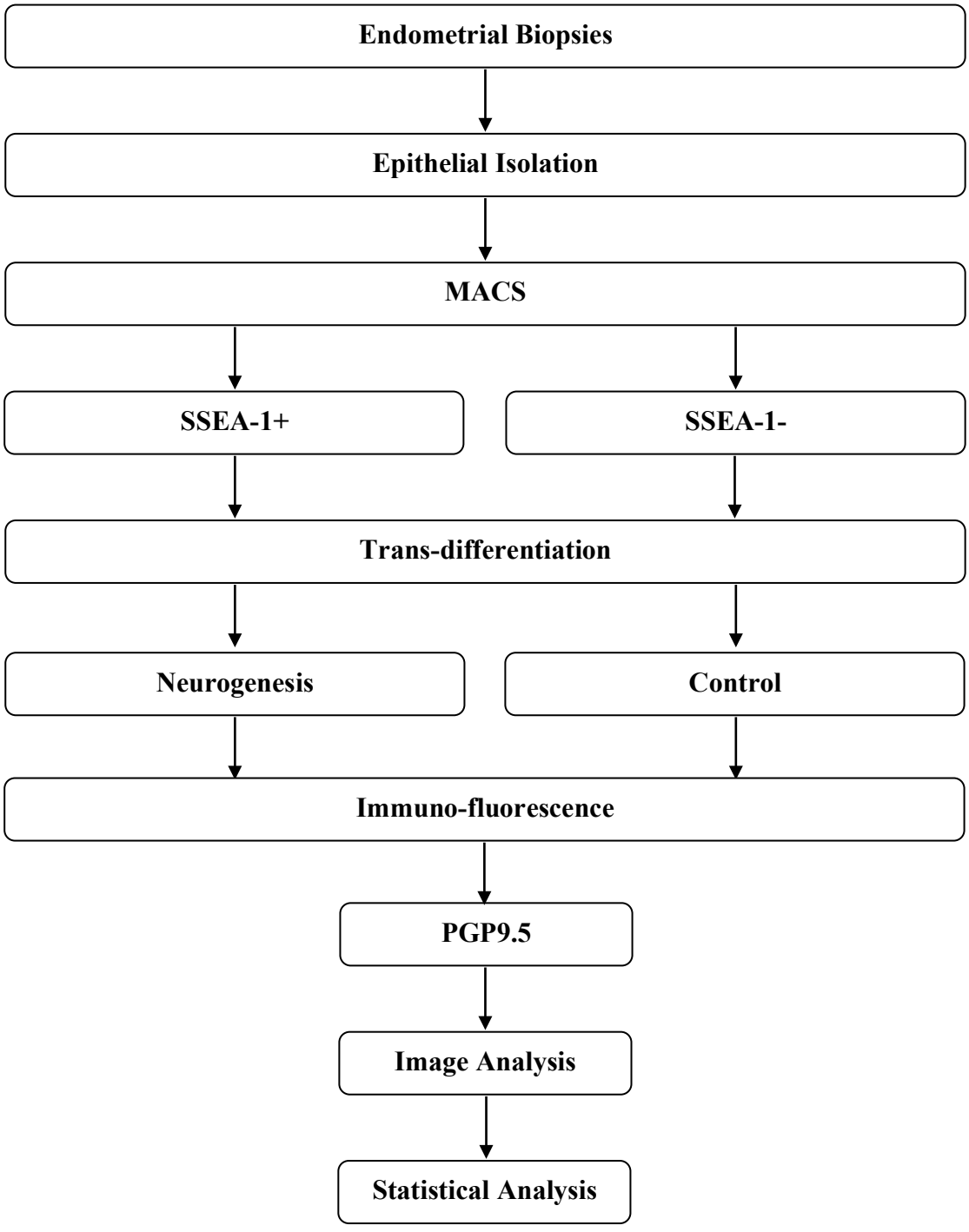

Figure 3.3: Flow diagram of study design to assess pluripotency of SSEA-1 enriched and depleted cell populations

3.2: Tissue Collection

3.2.1: Ethics approval

Collection of human endometrium was approved by Liverpool Adult Ethics committee (LREC references; 09/H1005/55 and 11/H1005/4). All samples were taken from women who had given informed and written consent (for letters of approval from the ethics committee, see Appendix I).

3.2.2: Study groups

Endometrial biopsies were taken from three different groups of patients. All participants were recruited at the Liverpool Women's Hospital (LWH).

Fertile Control Group

Inclusion Criteria

- Women aged between 18-51years
- Able to give informed voluntary consent
- Undergoing laparoscopy or hysterectomy for benign conditions at LWH

Exclusion Criteria

- Pregnant, currently/within the last three months
- Breastfeeding, currently/within the last three months
- Exogenous hormonal therapy within the last three months
- History of infertility
- History of endometrial pathology
- History of endometriosis

Endometriosis group

Inclusion criteria

- Women aged between 18-51 years
- Able to give informed voluntary consent
- Undergoing laparoscopy or hysterectomy for benign conditions at LWH
- Surgical diagnosis of active endometriosis

Exclusion criteria

- Pregnant, currently/within the last three months
- Breastfeeding, currently/within the last three months
- Exogenous hormonal therapy within the last three months

Post-menopausal group

Inclusion criteria

- Women aged over 51 years
- Able to give informed voluntary consent
- Undergoing laparoscopy or hysterectomy for benign, non endometrial conditions, such as uterine prolapse, at LWH
- Last menstrual period over 12 months ago

Exclusion Criteria

- Exogenous hormonal therapy within the last three months
- History of endometrial pathology

3.2.3: Identification and recruitment of participants

Theatre lists were scanned on a daily basis to highlight potential candidates. From there on, patient notes were studied and brief consultations with patients helped to clarify whether specific criteria were met. Only Good Clinical Practice (GCP) trained personnel took consent to ensure it was fully informed, competent and voluntary. Both verbal and written consent was obtained. Patient demographic data such as age, weight, height, body mass index, smoking history, parity, history of miscarriage and termination of pregnancy, cycle length, days of bleeding, LMP, reason for surgery and endometriosis stage (if relevant) were also collected from each participant (for examples of Patient Information, Consent and Data Sheets, see Appendix I). Surgeons were also asked to confirm the presence/absence of active endometriosis and stage (if relevant) at the time of performing the procedure.

3.2.4 Biopsy collection

Biopsies were taken in theatre to ensure the procedure was carried out in a sterile and safe manner. Only trained professionals took endometrial samples. Two types of samples were collected: full thickness and pipelles.

3.2.4i: Full thickness samples

Full thickness samples were collected when a hysterectomy was performed. Once the uterus was removed, it was immediately transferred to a surgical trolley with the anterior side facing downwards. A shallow incision was made in the midline from the fundus running inferiorly to the cervical canal on the posterior aspect (Figure 3.4, line **a**). This was repeated until the uterine cavity came into view, exposing the endometrial tissue. This task was assisted with the use of a surgical blade (size 22,

Swann-Mortem, Sheffield, UK) and Bonney Forceps (Thermo Fisher, Runcorn, UK). From there on, a second incision was made (**b**) to remove a full thickness section containing both myo- and endometrium. The endometrium was visualised and a final incision was made into the myometrium running approximately 1-2mm from the endo-myometrial junction (**c**). This section would be used for dating of the biopsy and immuno-histochemical analysis.

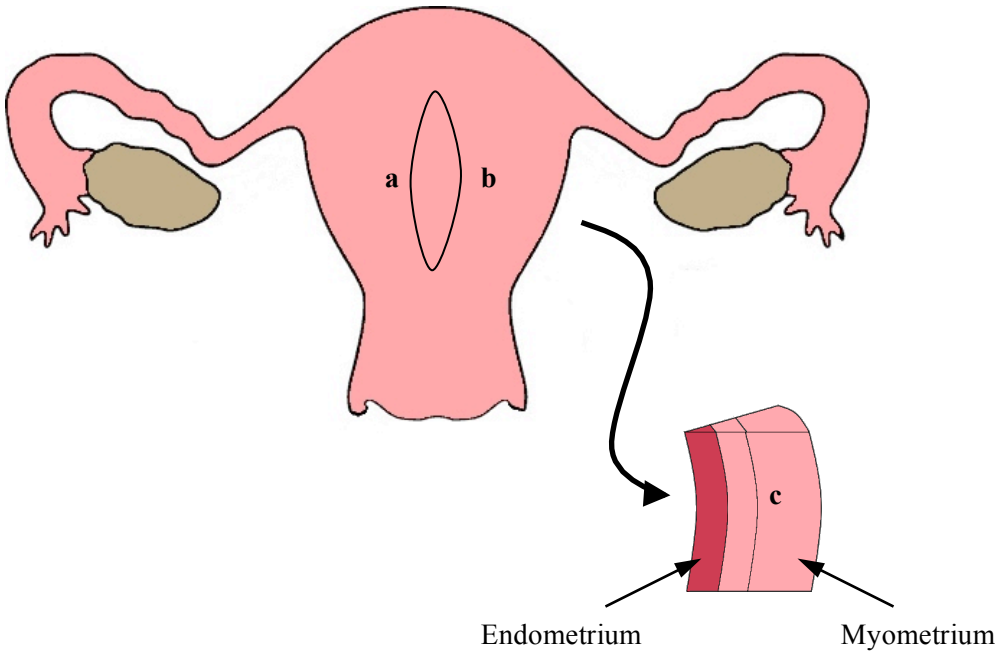

Figure 3.4: Full thickness samples. Full thickness samples were obtained by first opening the pelvic cavity **(a)**, a parallel incision was then made to withdraw a 'wedge' sample **(b)**. The endometrium was then visualised and removed via a final incision **(c)**.

For tissue culture and ribonucleic acid (RNA) extraction, a second approach was used. A blade was run along the inner aspect of the uterine cavity, removing any endometrial fragments. Care was taken not to disrupt the endo-myometrial junction, to avoid contamination with myometrial cells.

3.2.4ii: Pipelle samples

In cases when the uterus was not removed, pipelle biopsies were obtained. This involved the use of an endometrial sampler at the beginning of the operative procedure. Pipelle sampling is a very low risk procedure and normally carried out in an outpatient setting.[193] Briefly, patients were placed in the lithotomy position and a speculum was used to visualise the cervix. A tenaculum was applied to the anterior lip of the cervix, providing gentle traction and minimising the risk of perforation. The pipelle (Laboratoire C.C.D., Paris, France) was then introduced into the uterus via the cervical os and advanced until gentle resistance was felt. The inner piston of the pipelle was withdrawn, creating a vacuum effect and removing the endometrial lining (Figure 3.5). The pipelle was rotated 360°C and moved up and down the uterine cavity to obtain as much tissue as possible. It is thought that this method collects mostly the functional layer of endometrium. The contents of the pipelle sampler were utilised for RNA extraction, histochemical analysis and cell culture.

Figure 3.5: Pipelle. The Pipelle endometrial sampler was used to isolate endometrium from women not undergoing a hysterectomy.

3.2.5: Biopsy storage

For each participant, three samples were collected and stored differently, depending upon the intended purpose of that given sample. For example, for tissue culture, tissue was immediately transferred to a sterile universal containing 15ml of

collection medium (section 3.4.7 for media details) and placed on ice. Samples were stored at +4°C and prepped within 24 hours to increase cell yield and viability.

To prevent RNA degradation, tissue was immediately transferred to a cryovial containing 500µl of RNA*later*® (Applied Biosciences, Warrington, UK). RNA*later*® is a solution that aims to stabilise cellular RNA in unfrozen tissue samples before being stored at -80°C.

In order to prevent decay, putrefaction and autolysis of the dead tissue, tissue was directly transferred to a universal containing 10% neutral buffered formalin (NBF; Sigma-Aldrich, Poole, UK) for histochemical analysis.

3.3. Immuno-histochemistry

3.3.1: Fixation

Samples were fixed in the standard fixative 10% NBF (Sigma-Aldrich). NBF is thought to form cross-links with proteins and aldehydes within the cells, creating a gel-like substance. This enables the sample to retain its cellular constituents *in vivo,* yet also withstand the subsequent events of staining. The dimensions of all samples were small enough to enable sufficient penetration between 1 and 3 days in NBF at room temperature.

3.3.2: Processing

Tissues were processed via the use of an automated processing machine using standard protocol (Shandon Citadel 1000 Processor, Cheshire, UK). Tissue processing aims to dehydrate the sample and impregnates it with a solidifying medium, permitting the cutting of very thin sections. In this case, the replacement medium was paraffin wax (Histoplast PE, Thermo Scientific, Loughborough, UK). See Table 3.1 for the automated schedule of processing fixed endometrial samples.

3.3.3: Embedding

Tissue was embedded with paraffin wax using the Shandon Histocentre 3 Embedding Station. Paraffin embedding was preferred to frozen samples as tissue architecture and cell morphology is better retained. For further details, see "Embedding Samples" in Appendix II.

Table 3.1: Automated schedule of processing

Step	Solution	Time
1	4% NBF	45 minutes
2	60% Ethanol	1 hour
3	70% Ethanol	1 hour
4	90% Ethanol	1 hour
5	100% Ethanol	1 hour
6	100% Ethanol	1 ½ hours
7	100% Ethanol	2 hours
8	Xylene 1	1 hour
9	Xylene 2	1 ½ hours
10	Xylene 3	2 hours
11	Wax 1	2 ½ hours
12	Wax 2	3 ½ hours

3.3.4: Transferral of sample to slide

Consecutive sections were cut at 3µm thickness using Microm rotary microtome (Microm Ltd., London, UK) and were floated out onto a pre-warmed water bath at 40°C, until any creases in the paraffin had flattened out. Slides, which had already been coated with aminopropyl triethoxy saline (APES), were used to pick up floating sections and left to dry overnight at room temperature. For more details on APES coating of slides see "APES coating slides" in Appendix II.

3.3.5: Preparation of the slide

Before any form of staining took place, the slide was baked at 60°C for one hour or alternatively at 37°C overnight. This was to ensure the sample was fully adhered to the glass slide. However, care was taken to prevent 'over-baking', which would increase background staining when performing immuno-histochemical reactions. Once on the slide, samples needed to be removed of wax to allow sufficient penetration of the aqueous stain, whether chemical of immuno-reactive. Slides were de-waxed using a standard protocol. See Table 3.2 for reagents and incubation times.

Table 3.2: Reagent incubation times for de-waxing of slides

Step	Solution	Incubation Time
1	Xylene	10 minutes
2	Xylene	10 minutes
3	100% Ethanol	5 minutes
4	100% Ethanol	5 minutes
5	90% Ethanol	1 minute
6	70% Ethanol	1 minute
7	Water	

3.3.6: Antigen retrieval methods

The formation of cross-links during formalin fixation can mask antigen epitope sites, and reduce the affinity for the corresponding antibody paratope. For this reason, heat induced epitope retrieval was used as a pre-treatment to improve results. Accordingly, slides were added to boiling buffer in a stainless steel pressure cooker.

After 1 minute, the slides were rapidly cooled and washed in Tris-buffered saline (TBS) in preparation for standard immuno-histochemistry. Citrate 10mM (pH6.0) and Tris-EDTA (pH9.0) were the only buffers used. For details as to which buffer was used for each antibody see Table 3.4.

3.3.7: Standard immuno-histochemistry protocol

Immuno-histochemistry is a standard procedure that is commonly used to identify cell phenotypes based on a variety of markers. The concept relies on the 'lock and key' analogy of a specific antigen and antibody. In order to discuss the concept of immunohistochemistry, it is necessary to first consider the antibody. Antibodies are a group of proteins, also called immuno-globulins (Ig). They recognise a unique part of a foreign target/antigen (e.g. SSEA-1) and are made of basic structural units, including 2 heavy chains and 2 light chains to produce a Y shaped structure. There are five types of heavy chains: α, δ, ε, γ and μ. They are classified as IgA, IgD, IgE, IgG and IgM on account of which heavy chain the Ig possesses. Each tip of the Y shaped structure contains a paratope that is specific for one particular epitope on an antigen. This key and lock analogy allows structures to bind together with high specificity.

In this study the two-step polymer-based approach was used. The method involves the addition of a primary antibody to a complementary antigen on the cell surface (Figure 3.6) The second step requires the use of a secondary antibody conjugated to an enzyme polymer, in this case Horse Radish Peroxidase (HRP). When the substrate 3'3-Diaminobenzidine (DAB) is finally added, the HRP enzyme produces an insoluble brown precipitate making it visible on the slide.

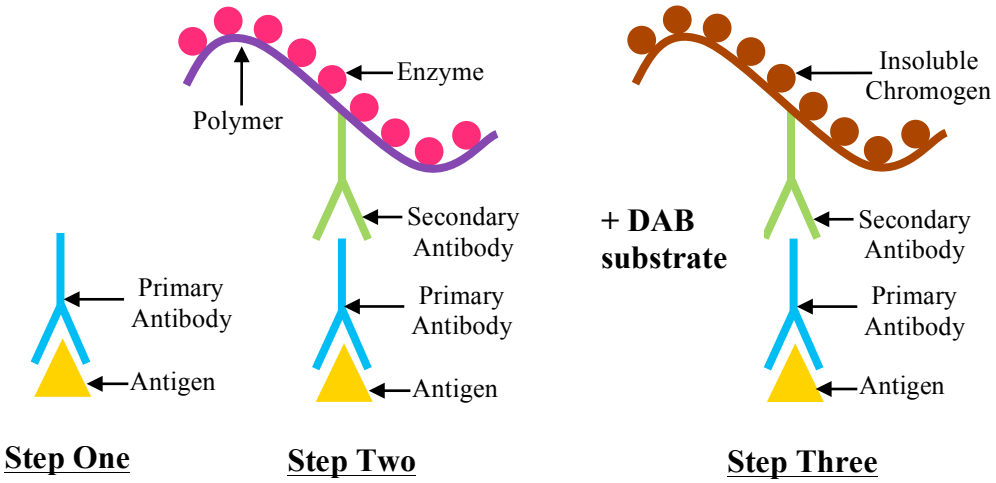

Figure 3.6: Immuno-histochemistry. A primary antibody is first applied, complementary to the antigen of choice. A secondary antibody conjugated to an enzyme polymer in then applied. When DAB substrate is finally added, the enzyme produces an insoluble brown precipitate.

Since this method requires use of HRP, pre-treatment with 0.3%H_2O_2/TBS (Sigma-Aldrich) for 10 minutes at room temperature was required to quench endogenous peroxidase activity. Slides were then washed in TBS before being marked with a hydrophobic pen, ensuring all tissue was surrounded with enough space to allow the antibody to spread. Slides were washed a second time in TBS and then incubated in horse serum block for 20 minutes (Vector Laboratories, Peterborough, UK) at room temperature to minimise background staining (if required). For details as to which antibody was blocked see Table 3.3.

Sections were then incubated with the primary antibody in a humidified chamber (for concentrations, incubation periods and temperature see Table 3.3). After incubation, slides were washed twice in TBS for 5 minutes and the polymer (ImmPACT kits, Vector Laboratories) from the same host was added for 30 minutes at room temperature. Once the polymer was removed; the slides were washed twice more in TBS for 5 minutes and the DAB substrate (ImmPACT, Vector Laboratories) was

added and left to incubate at room temperature in a humidified chamber. The reaction was stopped after 10 minutes by immersing the slides in H_20.

3.3.8: Antibodies

Table 3.3: Antibodies, concentrations, incubation times and supplier details

Antibody	Host Species	Clone	Horse Serum	Concentration	Incubation Time	Incubation Temperature	Catalogue Number	Supplier Details
ER-α	Rabbit	N/A	Yes	1:50	2 hours	RT	S1353	Epitomics, Suffolk. UK
ER-β	Mouse	PPG5/10	No	1:50	O/N	+4°C	MCA1974S	Serotec, Oxford, UK
PR	Mouse	PgR 636	No	1:1000	30 mins	RT	M3569	Dako, Cambridge, UK
CK18	Mouse	DC10	No	1:200	30 mins	RT	M7010	Dako, Cambridge, UK
β-Catenin	Rabbit	N/A	Yes	1:400	O/N	+4°C	9582	Cell Signalling, Herts, UK
***pan*Actin**	Mouse	AC-40	No	1:500	O/N	+4°C	A4700041 M4817	Sigma-Aldrich, Dorset, Poole
NANOG	Rabbit	D73G4	Yes	1:800	O/N	+4°C	4903	Cell Signalling, Herts, UK
OCT4a	Rabbit	C30A3	Yes	1:50	O/N	+4°C	2840	Cell Signalling, Herts, UK
SOX2	Rabbit	D6D9	Yes	1:50	O/N	+4°C	3579	Cell Signalling, Herts, UK
PODXL	Mouse	222328	No	1:100	1 hour	RT	MAB1658	R&D Systems, Oxford, UK
SSEA-1	Mouse	MC-480	No	1:800	O/N	+4°C	125602	Biolegend, London, UK
CK5/6	Mouse	D5/16B4	No	1:350	O/N	+4°C	MAB1620	Millipore, Durham, UK
H2AX	Mouse	3F2	No	1:12000	30 mins	RT	Ab22551	Abcam, Cambridge, UK
8-OHdG	Mouse	N45.1	No	1:200	1 hour	RT	Ab48508	Abcam, Cambridge, UK
Telomerase	Rabbit	N/A	Yes	1:2000	2 hours	RT	Ab27573	Abcam, Cambridge, UK
Ki67	Mouse	MM1	No	1:200	2 hours	RT	MM1LCE	NovoCastra, Newcastle, UK
M30	Mouse	M30	No	1:75	O/N	+4°C	2140322	Roche, Sussex, UK
CPP32	Mouse	JHM62	Yes	1:25	O/N	+4°C	NCLCPP32	NovoCastra, Newcastle, UK

3.3.9: Controls

In order to establish whether the experiment had been successful, a positive control was used for each antibody. Likewise, a negative control was incorporated into each run to verify that the staining was specific. In this case, an IgG from the same host species at the same concentration was used.

Table 3.4: Antigen retrieval methods, positive controls and anticipated marker

Antibody	Antigen Retrieval	Positive Control tissue	Anticipated Marker
ER-□	Citric acid	Breast cancer	Oestrogen dependency
ER-□	Citric acid	Secretory endometrium	Oestrogen dependency
PR	Citric acid	Proliferative endometrium	Progesterone dependency
CK18	Citric acid	Endometrium	Urogenital epithelia
□-Catenin	Citric acid	Endometrium	Epithelia
***pan*Actin**	Citric acid	Endometrium	Polarity (apical)
NANOG	Citric acid	Endometrial carcinoma	Undifferentiated state
OCT4a	Citric acid	Endometrial carcinoma	Undifferentiated state
SOX2	Citric acid	Endometrial carcinoma	Undifferentiated state
PODXL	Citric acid	Kidney	Undifferentiated state
SSEA-1	Tris/EDTA	Kidney	Undifferentiated state
CK5/6	Tris/EDTA	Breast	Undifferentiated state
H_2AX	Citric acid	Skin	DNA damage
8-OHdG	Citric acid	Colorectal carcinoma	Oxidative stress
Telomerase	Citric acid	Tonsil	Prolonged self-renewal
Ki67	Citric acid	Tonsil	Proliferation
M30	Citric acid	Colorectal carcinoma	Apoptosis
CPP32	Citric acid	Tonsil	Apoptosis

3.3.10: Haematoxylin staining

Sections were counterstained with haematoxylin to allow for full histological analysis. Haematoxylin is a basophilic nuclear stain that is blue/black in colour. Once oxidised, it forms haematein and has a high affinity to nuclear acids within the nucleus. Slides were dipped into Gills 2 haematoxylin (Thermo Scientific) for 1 second and then rapidly transferred to H_20. They were then de-hydrated using standard methods and mounted with consul-mount (Thermo Scientific). In addition, all patient samples used in the study routinely underwent haematoxylin and eosin (H&E) staining to establish cycle stage (for more details see Appendix II).

3.3.11: Dating of the biopsy

Despite the LMP being recorded from each participant, the possibility of recall bias and cycle irregularity meant that this method was ill equipped at accurately predicting cycle stage. An alternative method was used, which required routine histological analysis. Samples were dated in accordance with Noyes criteria and assessed independently by a blinded consultant gynaecologist and a gynaecological pathologist.[194] Those biopsies in perfect agreement between the two were used for the study.

3.3.12: Image analysis

Microscope slides were visualised and captured using Nikon Biophot Microscope and camera head (Nikon, Tokyo, Japan). Images were analysed using ImageJ software (version 1.46). Positive cells were counted and given as a proportion of total cells in polarised glands-like structures, generated from 3D culture. 10 *in vivo* glands from each of the following categories were analysed in the same way; pre-

menopausal functional and basal eutopic endometrium at the WOI from patients with and without endometriosis, and PM endometrium. The basal layer of the endometrium was defined as 300μm above the endo-myometrial interface and the functional layer was the area from the epithelial surface to the basal layer.[60]

3.4 Cell Culture

3.4.1: Epithelial cell isolation

As discussed previously the endometrium is comprised of epithelia and stroma. Separation of these cell types was achieved by mechanical and enzymatic digestion. Under culture conditions, stromal cells are bipolar and have an elongated shape. Epithelial cells are more polygonal in appearance and grow in discrete patches (Figure 3.7).

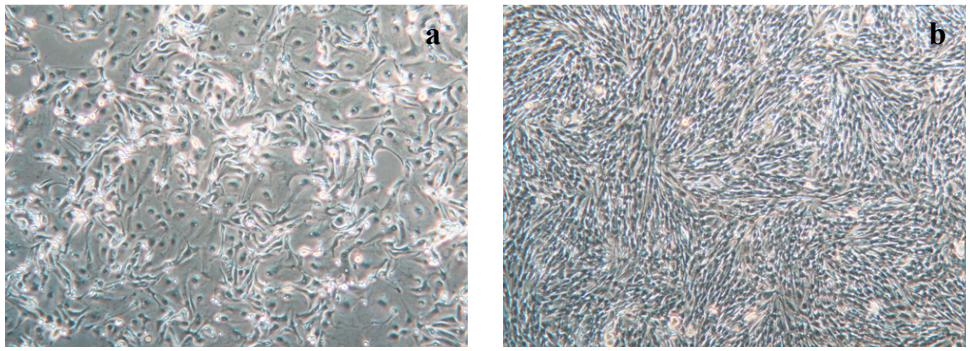

Figure 3.7: Endometrial cells in culture; Epithelia **(a)**; Stroma **(b)**

This separation technique relies largely on two principles;

1. After mechanical/enzymatic digestion, epithelia exist in clusters due to their stronger cell-cell junctional bonds, whereas stromal cells can easily be separated into single cells.
2. Stromal cells adhere to plastic surfaces more readily than the epithelial cells and survive in isolation.

Based on these principles when filtrated, stroma pass through and epithelia are retained (Figure 3.8). The fractions are further enriched by selective adherence.

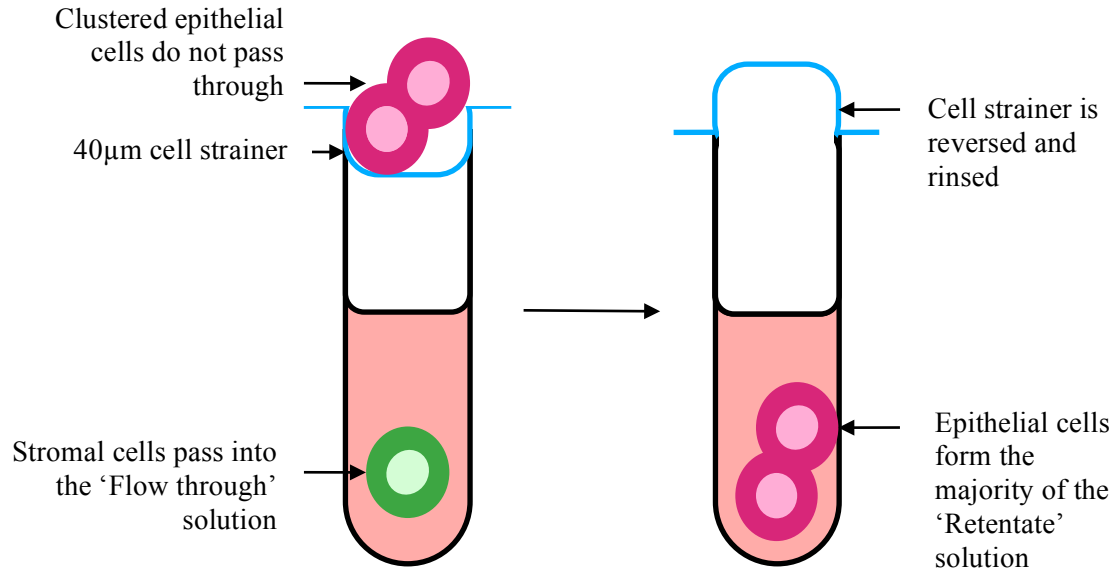

Figure 3.8: Separation of endometrial epithelia and stroma. After mechanical and enzymatic digestion, cells are filtered through a 40µm strainer; stromal cells pass through whereas epithelial cells remain clustered and are retained.

In order to obtain the epithelial fraction, the biopsy was first transferred to a petri-dish. Two scalpel blades (size 22, Swann-Mortem) were used in a criss-cross manner to chop the sample until it resembled the consistency of a fine mince. The tissue fragment suspension was then transferred to a 30ml universal tube and centrifuged for 5 minutes at 500g at 4°C. The pellet was re-suspended in 4ml of DMEM (Sigma-Aldrich) and incubated with 100µl DNase 1, 4mg/ml, (Roche) 500µl collagenase, 20mg/ml, (Invitrogen, Paisley, UK) and 500µl dispase, 10mg/ml, (Invitrogen) in a shaking water-bath for 90 minutes at 37°C. Afterwards, the cell suspension was filtered through a 40µm cell strainer (VWR Jencons, Leicestershire, UK) into a 50ml tube. The flow through solution was presumed to be the stroma and discarded. The cell strainer was then backwashed with more DMEM into a second 50ml tube (VWR). The backwash retentate was believed to be enriched with epithelia and then spun at 500g for 5 minutes at 4°C.

Glandular epithelia occur in clusters; the initial step of enzymatic digestion fails to separate the epithelia into single cells. In order to generate a single cells suspension, the cell pellet was re-suspended in 1ml of DMEM and an equal volume of 0.25% trypsin/EDTA (Sigma-Aldrich) was added plus 100µl DNase 1, 4mg/ml (Roche). The suspension was incubated for 20 minutes at 37°C. Afterwards, the trypsin reaction was stopped with DMEM and centrifuged for 5 minutes at 500g. The pellet was finally re-suspended and plated in a 100mm dish, in a humidified incubator for 20 minutes to enable selective adherence.

To prevent stromal contamination, the principle of selective adherence was applied. Due to the adhesive nature of stroma, most contaminating cells stick down to the surface of the dish quickly. After twenty minutes the media was removed from the dish and transferred to a new 100mm dish. New media was added to the already adherent culture. A judgement was made whether the first/second plate had a higher population of epithelial cells with fewer stromal contamination based on morphological appearance.

3.4.2: Magnetic cell sorting on SSEA-1

SSEA-1 is cell surface glycan. For this reason, cells expressing this marker can be isolated from a cell suspension via MACS. Before discussing the methodology it is important to appreciate the mechanism behind the technique. MACS relies on three components; microbeads, a separator and a column. Microbeads are super-magnetic particles, roughly 50nm in diameter. In this study, direct magnetic labelling was used; an antibody was directly attached to a magnetic microbead. Because microbeads are composed of iron oxide and polysaccharides, they are bio-degradable

and disappear from cells within a few days: they have minimal effect on cellular structure, function or activity.

Once incubated together, antibody specific magnetic beads bind to appropriate cells. The separator is a strong permanent magnet and induces a high gradient magnetic field upon the MACS column. When the cells are passed through the column, magnetically labelled cells are retained; unlabelled cells pass through.

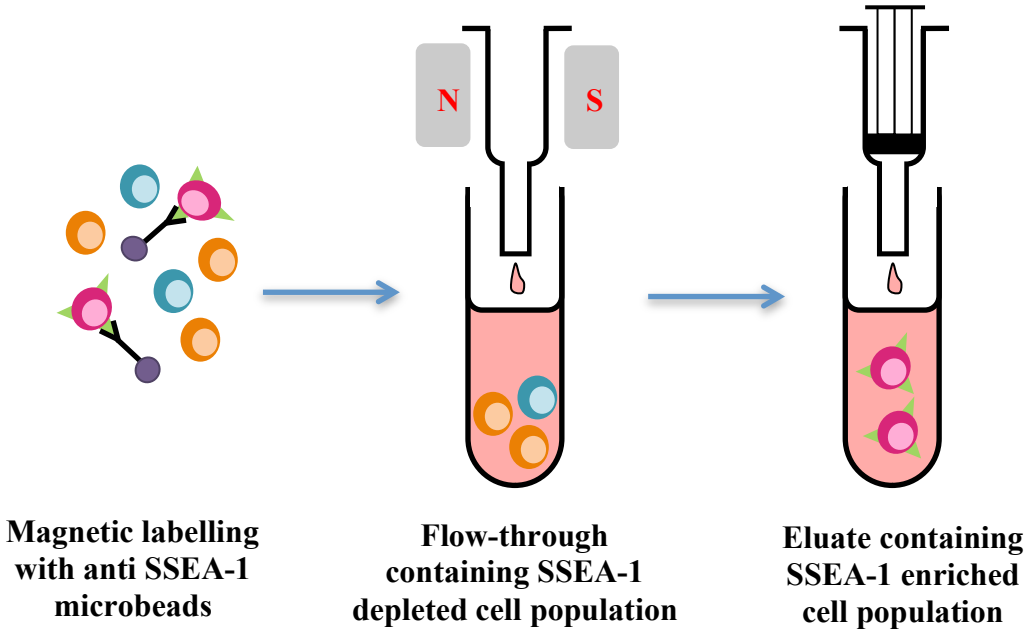

Figure 3.9: MACS on SSEA-1

When the column is removed from the magnet, the magnetic field is lost. With pressure, the labelled cells pass through and can be collected. In this study both labelled and unlabelled cells were utilised. The advantage of MACS over other cell selection methods is the limited time-frame the cells are in suspension and consequently increased cell viability and yield.

Cells were sorted between four and seven days after sample collection. Data from our laboratory (not shown) has shown that SSEA-1 expression peaks at this point. Media was first aspirated and cells were washed in sterile phospho-buffered saline (PBS; Sigma-Aldrich). 0.25% of trypsin/EDTA (Sigma-Aldrich) was added and cells were incubated for 5 minutes at 37°C in a humidified incubator. To stop the trypsin reaction, an equal volume of *epithelial media* was added (for list of media reagents see section 3.3.7). Cells were filtered through a pre-separation filter (Miltenyi Biotec, Surrey, U.K.) into a 50ml tube to remove any clusters of cells, which may block the column.

The flow through was centrifuged at 500g for 5 minutes. Once centrifuged, the pellet was re-suspended in 1ml of MACS buffer and transferred to a microfuge tube (for full details on MACS buffer reagents, see 3.3.8). This was then spun for 3 minutes at 700g. The pellet was re-suspended in 80µl of buffer and 20µl of anti-SSEA-1 microbeads (Miltenyi Biotec) and incubated for 30 minutes at 4°C. 1ml of buffer was then added to the suspension and spun at 700g for 5 minutes. The pellet was washed in MACS buffer again before finally being suspended in 0.5ml of MACS buffer.

The column (Miltenyi) was first prepared by filtering through 0.5ml of MACS buffer. The loading sample was filtered and collected into a microfuge tube and placed on ice. The filter was washed twice more with 0.5ml of MACS buffer to remove and collect anymore SSEA-1^- cells until a third wash was performed and discarded. The column was removed from the magnet and 1.5ml of *epithelial media* was added and forced with a plunger through the column. This was the eluate: enriched in SSEA-1^+ cells. Both fractions were then counted using a Neubaur

Haemocytometer (CamLab Ltd., Cambridge, U.K.) and seeded at a density of 20,000 cells/cm^2 into 24 well plates (VWR) and 8 well chamber slides (BD Biosciences, Oxford, UK).

3.4.3: Multi-lineage differentiation

3.4.3i: Adipogenic assay

The formation of adipocytes from MSCs is a complex process. The first phase, known as determination involves the commitment of a MSC to a 'pre-adipocyte'. A pre-adipocyte is indistinguishable from its precursor cell, but it has lost its ability to differentiate into other cell types. The second phase, known as terminal differentiation, involves the accumulation of the typical 'fat cell' appearance.[195] This involves the conversion of the fibroblast to a spherical shape. The progression is completed when the cells gain insulin sensitivity and the ability to synthesise and transport lipids. The cells accumulate lipid vacuoles that can be stained positive by Oil Red O. Adipocytic markers include peroxisome proliferation-activated receptor gamma2 (PPARγ2), said to be the 'master of adipogenesis'.[195] The CCAAT-enhancer binding proteins (C/EBP) are also integral to development. Lipoprotein (LPL) is an early marker of differentiation; whereas adipocyte protein 2 (aP2) and adiponectin are expressed during the latter stages. Adipogenesis requires certain reagents, namely dexamethasone, 3-isobutyl-1-methyl-xanthine (IBMX), indomethacin and insulin. Dexamethasone is a glucocorticoid and induces expression of C/EBP and PPARγ. IBMX aims to amplify the effects of dexamethasone and prevents osteogenic differentiation. Indomethacin has a similar function and insulin appears to be the most potent 'adipogenic-inducer' by increasing triglyceride formation.[196]

Accordingly, confluent cells were stimulated with *adipogenic media* every 2-3 days (for full details on *adipogenic media,* see section 3.3.7). Non-stimulated cells were cultured in *epithelial media* and served as a negative control. After two weeks, cells were washed twice with PBS and fixed by incubation with 4% para-formaldehyde (PFA; Sigma-Aldrich) for 10 minutes for staining analysis.

Oil Red O staining was used to confirm the presence of lipid vacuoles. Briefly, PBS was removed from fixed cells and replaced with 60% Isopropanol (Sigma-Aldrich). After 10 minutes, 60% Oil Red O stain solution was added and left for another 10 minutes until washed with water (for full details on Oil Red O solution see section 3.3.8). Cells were counterstained with Gills 2 haematoxylin (Thermo Scientific). Images were visualised and captured with the use of a Nikon Biophot Microscope and camera head (Nikon).

3.4.3ii: Osteogenic assay

The determination of osteoblastic lineage is also two-fold and reliant upon hormones and transcription factors.[197] Initially the expression of Runt- related transcription factor-2 (Runx-2) directs a mesenchymal stem cell into osteoblastic lineage. This is assisted by the effect of dexamethasone, which is also instrumental at collagen expression in the ECM. Care needs to be taken when using dexamethasone as high concentrations can induce osteoporosis *in vivo*. β-catenin molecules and Osterix (OSX) allow for the further development into an immature osteoblast. Cells become less spindle-like and the ECM becomes mineralised with the addition of calcium and phosphate ions. β-glycerophosphate (β-GP) acts as a source of these ions and

alkaline phosphatase (ALP) expression increases to free inorganic phosphate. Early markers of osteoblastic differentiation comprise ALP and osteonectin. Osteocalcin (OC) and osteopontin (OP) are expressed later in the differentiation process.

Near confluent cells were stimulated every 2- 3 days with *osteogenic media* for two weeks (for media details, see 3.3.7). Cells were stimulated with *epithelial media* for the same period and served as a negative control. On day 15, cells were fixed by incubation with 4% PFA at room temperature for 10 minutes and subject to analysis. Visual analysis consisted of ALP staining. Accordingly, PBS was aspirated from fixed cells and replaced with 400µl of Fast Red/Napthol solution. (Sigma-Aldrich) and left to incubate at room temperature. After 30 minutes, cells were rinsed with 0.1MTris HCl (pH 9.2) and PBS before being counterstained with 4',6-diamidino-2-phenylindole (DAPI).

3.4.4: hMSC cell culture

Human mesenchymal stem cells (hMSCs) were used as the positive control for *in vitro* differentiation into adipocytes and osteocytes (Lonza, Walkersville, Inc., USA).

3.4.4i: Cell thawing protocol

hMSC were thawed by removing cell containing cyrovials from a liquid nitrogen tank. The cryovial was promptly transferred to a waterbath at 37°C. As soon as the cell suspension thawed, it was transferred to a 15ml tube, pre-filled with warmed hMSC Growth Medium^{TM} (hMSCGM; Lonza, Walkersville Inc., USA). Subsequently, the cells were centrifuged at 300g for 3.5 minutes and the supernatant

discarded. The cell pellet was re-suspended in hMSCGM and transferred to a dish, before being placed in a humidified incubator at 37°C, 5% CO_2 in air.

3.4.4ii: Cell passaging

In order to passage cells, culture medium was aspirated. The cells were washed twice in sterile PBS. Next, cells were incubated with 0.25% trypsin/EDTA (Sigma-Aldrich) for 2 minutes in a humidified incubator. The trypsin reaction was then stopped with an equal volume of pre-warmed hMSCGM (Lonza). The cell suspension was transferred to a 15ml tube and spun at 300g for 2.5minutes. Cells were re-suspended in 1ml hMSCGM before being counted using a Neubauer Haemocytomer (CamLab). Usually cells were passaged in a 1 to 2 ratio and were expanded up to passage 7.

3.4.4iii: Routine hMSC culture

Cells were cultured according to manufacturer's instructions. Briefly, hMSCs were maintained in hMSCGM on uncoated dishes. Media was changed every 2-3 days. Cells were passaged when they reached 90% confluency, roughly every week. Cells were seeded according to manufacturer's instructions at 5,000-6,000 cells per cm^2 of surface area.

3.4.5: Pluripotency assay (Neurogenic trans-differentiation)

Cells were plated onto pre-coated Matrigel^{TM} chamber slides to increase cell viability. Once cells had reached near confluence, *epithelial media* was aspirated and replaced with *neurogenic media*, kindly donated by Dr. D Wilkinson, University of Liverpool. Media was changed every 3-4 days.

After 10 days cells were fixed with 4% PFA and subjected to immuno-fluorescence using a standard technique. Cells were initially permeabilized with 0.1% (v/v) Triton X-100/PBS (VWR) for 10 minutes at room temperature. After 3 washes with PBS, cells were blocked with 1% (v/v) BSA/10%normal goat serum/PBS for 1 hour at room temperature, prior to overnight incubation with rabbit anti-protein gene product 9.5 antibody (PGP9.5; AbD Serotec, Product Number 7863-0504, Oxfordshire, UK), at a concentration of 1:1000. The primary antibody was subsequently washed off and cells were incubated with fluorescein isothiocyanate (FITC)- conjugated anti-rabbit antibody (Cell Signalling) for 1 hour at room temperature before being rinsed with PBS.

3.4.6: 3D cell culture

3D culture required the use of Matrigel^{TM} (BD Biosciences). SSEA-1 enriched fractions have been found to have a greater propensity to produce organoid/gland-like structures than SSEA-1 depleted counterparts (1.4.7). However, a real difficulty is the small yield that is generated, due to prolonged culture and sorting. For this reason, crude epithelial cells were used in this assay, as it is presumed the structures will be from SSEA-1 origin.

3.4.6i 3D culture of endometrial epithelial cells

Following epithelial isolation (Section 3.3.1i) cells were cultured until confluency. At this point, cells were trypsinised and centrifuged at 5g for 5 minutes. The pellet was re-suspended in 1ml of *serum free media* (for media details see 3.4.7) and transferred to a microfuge tube. The pellet was spun once more at 700g for 5 minutes and re-suspended in Matrigel^{TM} at a concentration of $5x10^3$ cells/ undiluted 1ml

Matrigel™. All work was done on ice. 50µl of cell-Matrigel™ suspension was placed in the centre of a 24-well-plate well and incubated at 37ºC. Matrigel™ hemispheres set after 20 minutes and 500µl of *serum free media* (see section 3.3.8 for details) was added. Media was changed every 2 – 3 days.

3.4.6ii: Harvesting

Matrigel™ hemispheres were harvested after 10-14 days dependent upon cell growth. 3D cultures were fixed in 10% NBF (Sigma-Aldrich) for 30 minutes, harvested into 1% (w/v) agarose/PBS and placed in NBF overnight at 4°C. 3D cultures were processed and sectioned as previously described (section 3.3.2 onwards).

3.4.7: Culture media reagents

Media was prepared in a sterile manner, and filtered using a 22µm filter.

Collection media

- DMEM (Sigma-Aldrich)
- 1% (v/v) Foetal Bovine Serum (FBS; BioSera Ltd, Sussex, UK)
- 0.2% (v/v) Primocin (Bioscience Lifesciences, Nottingham, UK)

Epithelial media

- High glucose DMEM/F12 (Lonza)
- 2% (v/v) L-glutamine (Sigma-Aldrich)
- 0.2% (v/v) Primocin (Sigma-Aldrich)
- 10% (v/v) FBS (Biosera)

- 200ng/ml recombinant human epithelial growth factor (EGF; Sigma-Aldrich)

Adipogenic media (*Gargett et al.,* 2009)[143]

- High glucose DMEM/F12 (Lonza)
- 0.2% Primocin (Bioscience Lifesciences)
- 500μM IBMX (Sigma-Aldrich)
- 1μM Dexamethasone (Sigma-Aldrich)
- 10μM Insulin (Sigma-Aldrich)
- 200μM Indomethacin (Sigma-Aldrich)

Adipogenic media

- High glucose DMEM/F12 (Lonza)
- 0.2% Primocin (Bioscience Lifesciences)
- 500μM IBMX (Sigma-Aldrich)
- 1μM Dexamethasone (Sigma-Aldrich)
- 10μM Insulin (Sigma-Aldrich)

Osteogenic media (*Gargett et al.,* 2009)[143]

- High glucose DMEM/F12 (Lonza)
- 0.01μM Vitamin D3 (Sigma-Aldrich)
- 50μM L-ascorbic acid (Sigma-Aldrich)
- 10mM β-glycerol phosphate (Sigma-Aldrich)

Neurogenic media (*Almond et al.,* 2007)[198]

- Low Glucose (1mg/ml) DMEM (Invitrogen)
- 1% (v/v) FBS (Gibco, Paisley, UK)
- 1% N1 Supplement (Sigma-Aldrich)
- 2mM L-glutamine (Gibco)
- 2% (v/v) Chick embryo extract (Centrifuge 8000rpm & used supernatant)(Sera Laboratories Int., Horsted Keynes, UK)
- 20ng/ml Fibroblast Growth Factor 2 (FGF2; Sigma-Aldrich)
- 0.5mM Mercaptoethanol (Sigma-Aldrich)
- 20ng.ml EGF (Sigma-Aldrich)
- 100U/ml Penicillin (Gibco)
- 100 μ g/ml Streptomycin (Gibco)

Serum-free media

- DMEM/F12 (Lonza)
- Insulin-transferrin-selenite (ITS; Invitrogen)
- 50ng/ml human recombinant EGF (Sigma-Aldrich)

3.4.8: Buffers and solutions

MACS buffer

- Culture grade PBS ($Ca^{2+/}Mg^{2+}$ free) (Sigma-Aldrich)
- 5% (v/v) FCS (Sigma-Aldrich)
- 2% (v/v) $EDTANa_2$ (VWR, Leicestershire, UK)

- Final solution was filtered through a 22µm mesh.

4% (w/v) Paraformaldehyde

- 4g Paraformaldehyde (Sigma-Aldrich)
- 100ml PBS (Sigma-Aldrich)

0.5% (w/v) Oil Red O solution

- 0.5g Oil Red O (Sigma-Aldrich)
- 100ml Isopropanol (Sigma-Aldrich)
- Prior to use the working solution was diluted in a 3:2 ratio with H_20 and filtered using Whatman paper

Fast Red/Napthol

- 2mg Napthol AS-MX phosphate (Sigma-Aldrich)
- 10mg Fast Red TR (Sigma-Aldrich)
- 10ml 0.1 TrisHCl (pH 9.2)

3.5: Molecular Biology

3.5.1: RNA extraction

Total RNA was extracted using TRIzol® reagent (Invitrogen) according to manufacturer's instructions. Briefly, culture medium was aspirated and cells were washed with PBS. PBS was then discarded and 250µl of TRIzol® was added to each well (in a 24 well plate). The solution was triturated with a pipette to produce a homogenous solution. Technical replicates were pooled to increase total RNA yield and transferred into a 1.5ml microfuge tube. A fifth of the total TRIzol® volume of chloroform (Sigma-Aldrich) was then added. Suspensions were shaken for 15 seconds and subsequently transferred to a centrifuge, spun at 12,000g for 15 minutes at 4°C. Following centrifugation, three distinct phases formed: a lower pink phase (containing mainly protein), a white interphase (containing DNA), and a clear aqueous upper phase containing the RNA. As much of the clear aqueous phase as possible was removed, taking care not to contaminate the sample with protein or DNA and each transferred to new microfuge containing 1µl of glycogen (Invitrogen). Half the original TRIzol® volume of Isopropanol (Sigma-Aldrich) was added and the solutions were inverted and left to incubate overnight at -20°C. Following incubation, solutions were centrifuged at 12,000g for 10 minutes at 4°C and the supernatant discarded. The pellet was washed in 75% EtOH (Sigma-Aldrich) and centrifuged for one last time at 12,000g for 8 minutes at 4°C. The supernatant was discarded and the pellet was allowed to air dry at room temperature. The pellet was then dissolved in 10-15µl of nuclease free water (Sigma-Aldrich), depending upon pellet size and stored at -80°C.

3.5.2: RNA quantification

The quantity and purity of total RNA was assed using NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher), according to manufacturer's instructions. The 260/280nm absorbance ratio, reflecting RNA purity, and concentration was recorded for each sample. Unfortunately, due to cell death and the small number of cells cultured, RNA quality and concentration were poor for some of the samples. Only the biological samples with the 'best full set' of RNA were used for quantitative polymerase chain reaction (qPCR). These were the samples with the highest concentration and a 260/280 value close to 2.

3.5.3: DNase treatment of total RNA

In order to prevent contamination of genomic DNA, treatment with deoxyribonuclease (DNase) enzymes was performed. 8µl of RNA was transferred to a 0.2ml microfuge tube and mixed with lµl of DNase buffer (Promega, Southampton, UK) and 1µl of DNase (Promega), and incubated at 37°C. After 30 minutes, 1µl of STOP buffer (Promega) was added and incubated at 60°C for 15 minutes. Samples were stored at -20°C.

3.5.4: cDNA synthesis

Complimentary DNA (cDNA) was acquired using the Superscript First-Strand Synthesis System. 4µl of DNase treated RNA were transferred to a 0.2ml microfuge tube along with 1µl of 100ng/µg stock solution of random hexamers (ABgene, Loughborough, UK), 1µl of 10mM dNTP mix (Bioline Ltd., London, UK) and 8µl of nuclease free water (Sigma-Aldrich). The solution was incubated at 65°C for 5 minutes and then placed on ice for 5 minutes. 4µl of 5X First-Strand buffer

(Invitrogen), 1µl of 0.1M DTT (Invitrogen) and 1µl of Superscript III (200U/µl; Invitrogen) were added, and the mixture was left to stand for 5 minutes at room temperature. Following this, the mixture was incubated for 60 minutes at 50°C, then inactivated at 70°C for 15 minutes.

3.5.5: Standard qPCR protocol

qPCR enables both detection and quantification of specific cDNA. It follows the principle of traditional PCR: the exponential amplification of cDNA due to thermal cycling. However, unlike conventional PCR, in qPCR the amplified product is measured throughout the experiment – not at the end point. Amplified DNA is detected via fluorescence; the earlier the fluorescence is detected, the greater the number of the product. In this study, the nonspecific SYBR® Green I dye was used, which becomes fluorescent once upon binding to double stranded DNA (dsDNA) (Figure 3.10).

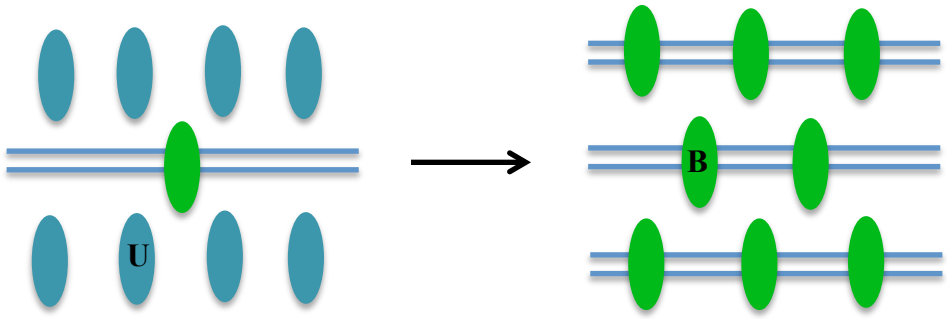

Figure 3.10: DNA-binding SYBR® Green I in qPCR. As amplification occurs, more dsDNA is available for SYBR® Green I to bind to. Bound SYBR® Green I is fluorescent; **(B)** bound SYBR; **(U)** unbound SYBR.

Before preparing the qPCR reactions, cDNA samples were diluted between two and three fold depending upon RNA concentration. The reaction was as follows; 10µl SYBR® Green Jumpstart™ Taq ReadyMix™ (Sigma-Aldrich), 7µl nuclease-free water (Sigma-Aldrich), 1µl cDNA and 1µl each of 0.25µM sense and anti-sense

primers (all purchased from Sigma-Aldrich). The reaction was carried out on the Rota Gene RG-300 real time cycler (Corbett, Sussex, UK) and required a 10 minute hold period, followed by 40 cycles of 95°C for 6 seconds, 60°C for 30 seconds and 72°C for 30 seconds. All primers had an annealing temperature of 60°C. Three technical replicates were run for each template and primer set. No cDNA template controls were included in all runs to ensure there was no contamination. A positive control was included to ensure the primer set was working successfully and calibrator samples were also included in each run to account for any inter-run variation. All gene expression was normalised to the endogenous reference gene YWHAZ, which is a protein implicated in general signalling pathways and is high in all cells.

Following each qPCR run, a melting curve was performed to ensure amplicons were the same for all biological and technical replicates. The temperature was set to increase 1°C every 5 seconds, from 75°C to 98°C. Peaks were representative of the temperature the dsDNA is dissociated. Amplicons of the same size and nucleotide composition demonstrate a common peak (Figure 3.11).

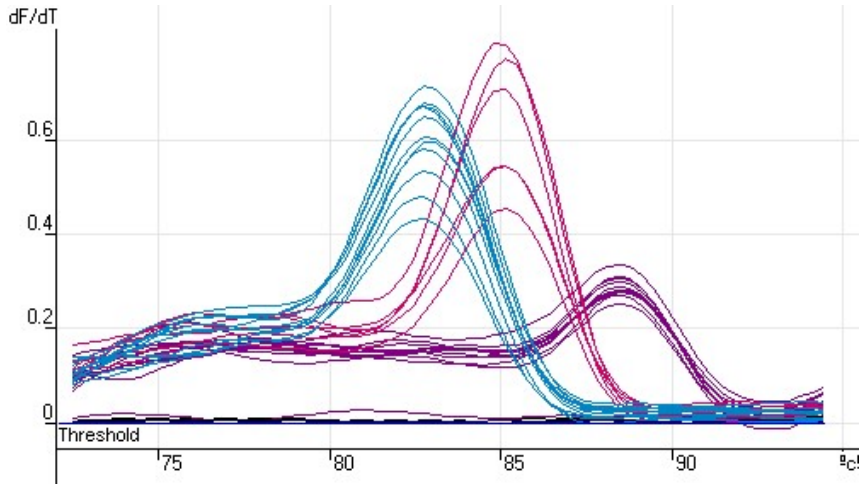

Figure 3.11: Melt curve of amplicons. Different coloured peaks represent 3 different amplicon products.

Results were collated using the Rotor Gene 3000 analysis software (version 6.1). The threshold was set to be both above the background fluorescence and also in the log-linear phase of the curve (Figure 3.12). The point at which the curves crossed the threshold was measured and defined as the C_T. C_T values were normalised to the endogenous reference gene and analysed according to the Pffafl method using the REST-2009 software (version 1).[199] Primer efficiency was assumed to be 1.0.

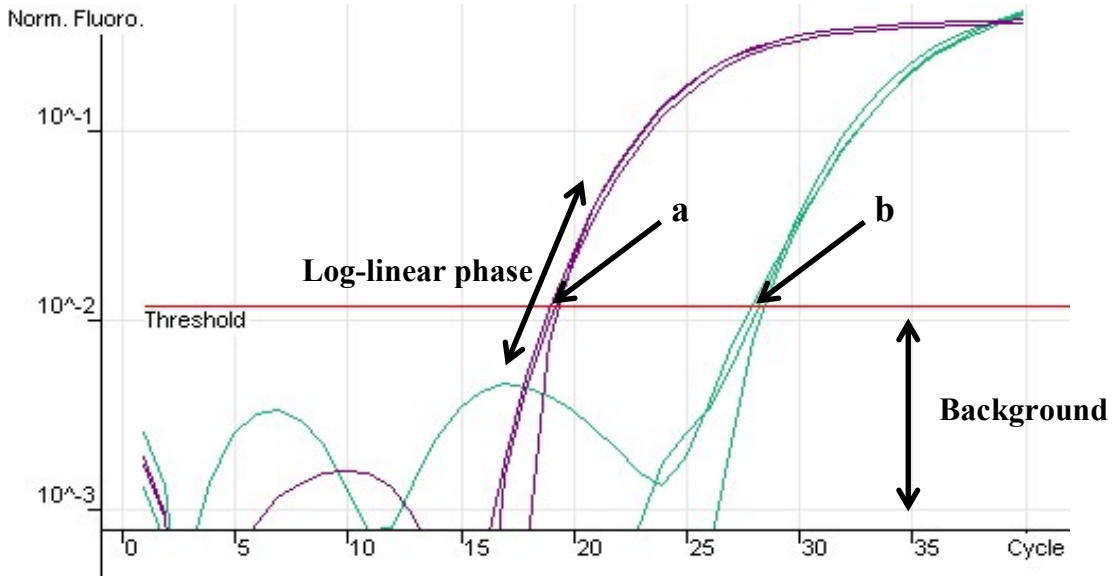

Figure 3.12: C_T values for target and reference genes. The threshold was set to cross the log-linear phase of the reaction and above any background; **(a)** represents the YWHAZ C_T value; **(b)** represents the reference gene C_T value.

3.5.6: Oligonucleotide primers

All primers were reconstituted accordingly in 1ml nuclease-free water (Sigma-Aldrich) to reach a working volume of 6.25pmol/µl. Aliquots were stored at -20°C until needed. The control for ADiPOQ, aP2, LPL and PPARγ2 were human mature adipocytes, kindly donated by Dr. C Bing, University of Liverpool. Likewise, Dr. D Barraclough, University of Liverpool, provided human osteoblastoma RNA as a control for ALP, RUNX2, OSX and OC (for full list of sequences, see Table 3.5).

3.5.7: Gel electrophoresis

Gel electrophoresis was conducted using the FlashGel™ System (Lonza, Vervieres, Belgium) using manufacturers instructions. Briefly, 1µl of amplicon, 1µl of loading buffer (Lonza) and 3µl of nuclease free water (Sigma-Aldrich) was added to each well and run at 250V for 5 minutes. Images were captured using the FlashGel™ camera (Lonza).

Table 3.5: Oligonucleotide primers. S: sense, AS: anti-sense

Primer	Sequence	Size	Source
hADiPOQ	S: AGCCTCCTTCTCCTGGGTCC AS: GTTGCCTCTAGCCTGGTGGG	295bp	*Yang et al.*, 2011[200]
haP2	S: ACCAGGAAAGTGGCTGGCAT AS: CAGGTCAACGTCCCTTGGCT	331bp	*Yang et al.*, 2011[200]
hLPL	S: GAGATTTCTCTGTATGGCACC AS: CTGCAAATGAGACACTTTCTC	276bp	*Yang et al.*, 2011[200]
hPPAR□2	S: TGTCTCATAATGCCATCAGGTTT AS: GATAACGAATGGTGATTTGTCTG	224bp	*Yang et al.*, 2011[200]
hALP2	S: GGACATGCAGTACGAGCTGA AS: CCAGCAAGAAGAAGCCTTTG	113bp	*Glennon-Alty* 2012[201]
hRUNX2	S: TCCTTAGAACAAATTCTGCCCTTT AS: TGCTTTGGTCTTGAAATCACA	136bp	*Wang et al.*, 2012[202]
hOSX	S: CCTCCTCAGCTCACCTTCTC AS: GTTGGGAGCCCAAATAGAAA	148bp	*Wang et al.*, 2012[202]
hOC	S: GAAGCCCAGCGGTGCA AS: CACTACCTCGCTGCCCTCC	70bp	*Glennon-Alty* 2012[201]
hYWHAZ	S: CGTTACTTGGCTGAGGTTGCC AS: GTATGCTTGTTGTGACTGATCGAC	69bp	*Marullo et al.*, 2010[203]
hACTB	S: AGTGTGACGTGGCACATCCGCA AS: GCCAGGGCAGTGATCTCCTTCT	112bp	*Marullo et al.*, 2010[203]

3.6: Statistical Analysis

Statistical analysis of gene expression was performed using the REST-2009 software (version 1).[199] Statistical analysis of neural differentiation was performed using the GraphPad Prims software (version 5). Since data were paired and n=3, the Wilcoxon test was used. P values <0.05 were considered statistically significant. Data are presented as mean ± SD unless otherwise specified.

Chapter Four:

OPTIMISATION AND

VALIDATION OF RESULTS

In order to improve experimental outcome, a number of optimisations took place for each technique. Examples are covered within this chapter.

4.1: Immuno-histochemistry

Most antibodies in this project had previously been optimised; this was not the case for β-catenin and *pan*Actin. Control sections were incubated with antibodies at various concentrations. The purpose of this was to find a balance between strong target, and background staining. Serial dilutions of the antibody were assessed; for β-catenin, 1:200, 1:400 and 1:800; for *pan*Actin, 1:250, 1:500, 1:1000 (Figure 4.1).

There were difficulties regarding the counterstaining of organoids. Standard methods typically over-stain sections with haematoxylin, only to 'de-differentiate with acid ethanol. The result is a clear image, one that easily enables visualisation of DAB products in cell structure. The acid alcohol treatment was noted to be harsh on the organoid sections, sometimes causing loss of the gland-like structures/organoids from the slides. This step was therefore omitted, necessitating a much shorter time in haematoxylin. The same incubation time was applied to whole tissue sections.

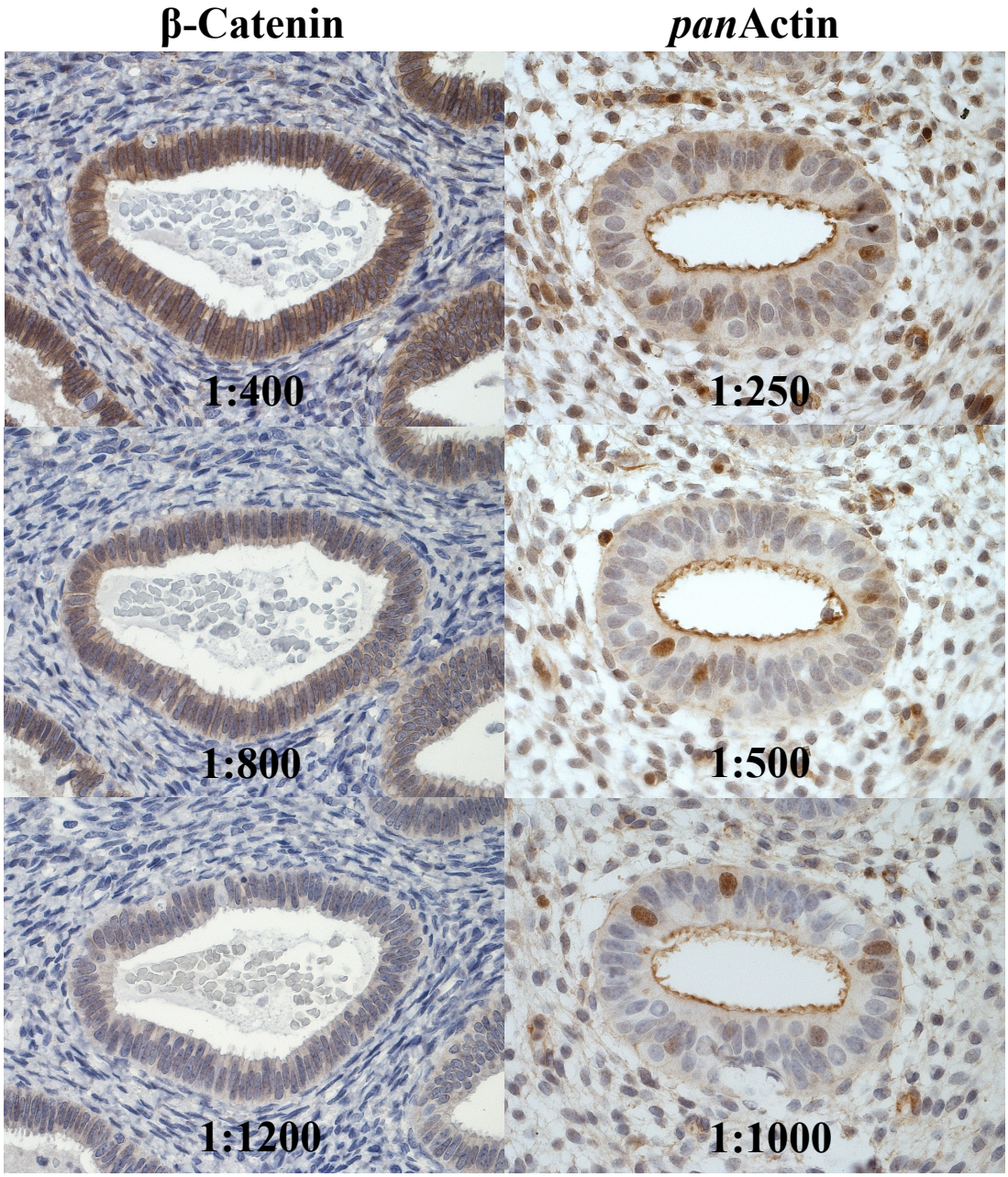

Figure 4.1: Optimisation for immuno-histochemistry
Serial dilutions for the antibodies β-Catenin and *pan*Actin were performed. A concentration of 1:400 for β-Catenin was chosen for future experiments. Likewise, a concentration of 1:500 for *pan*Actin was chosen.

4.2 Cell Culture

4.2.1: Multipotency

Initially, the adipogenic differentiation protocol was taken from *Gargett et al., 2009*.[143] The authors had successfully differentiated human ESt-ASPC into adipocytes and did not complain of any difficulties. However, when this media was added to human endometrial epithelial cells, it quickly became apparent that the media was promoting cell senescence and death. Three out of four biological replicates underwent apoptosis within 24 hours of incubation with this media; cells in control media were still viable. The only sample to survive more than 24 hours had an unusual phenotype; 'vacuoles' appeared to be present within the cell structure (Figure 4.2). These did not stain positive with Oil Red O, and are likely to be representative of cell membrane rupture. Modifications were made to the media, with the use of more dilute reagent concentrations. Indomethacin was completely removed from the protocol, as a thorough literature search found this had a deleterious effect on human endometrial epithelial cells.[204] hMSCs were seeded in triplicate and subjected to the same media, to validate the experiment.

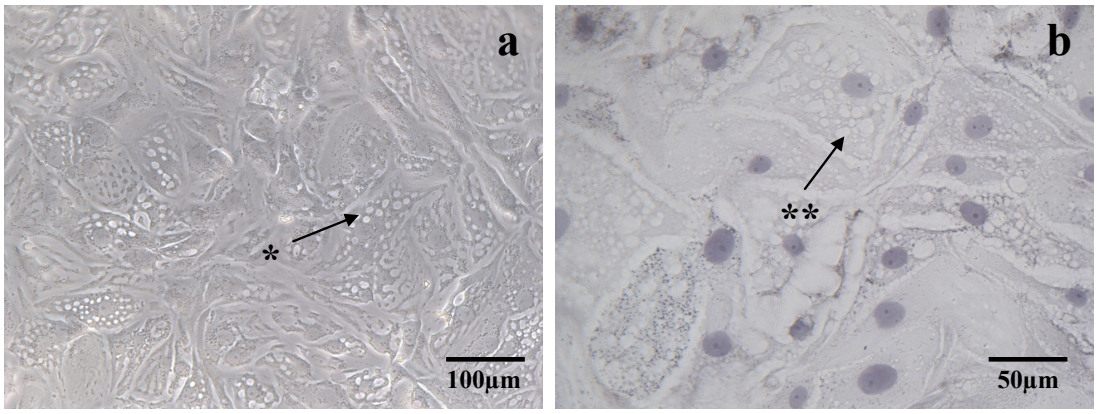

Figure 4.2: Optimisation of cell culture
Adipogenic induced SSEA-1 enriched and depleted fractions underwent cell death or produced large vacuoles **(a)**, see *. These were negative for for Oil Red O **(b)**, see **.

4.2.2: Pluripotency

For neurogenic assays, cells were initially cultured in 8 well chamber slides. However, it quickly became apparent that cell viability was poor. This was overcome by two strategies; firstly the protocol was shortened from 3 weeks to 7-10 days, and secondly, a film of Matrigel^{TM} was plated over the slide chamber base.

4.3: Molecular Biology

Before the commencement of qPCR reactions, reference genes were run on RNA, DNase-treated RNA, and cDNA for each sample set (Figure 4.3). The purpose of which, was to confirm genomic DNA had been degraded, and cDNA synthesis had been effective. DNase-treated RNA should have the highest C_T value (less content); RNA should have a slightly lower value as it contains some genomic material. However, cDNA should have the lowest value and will be detected first.

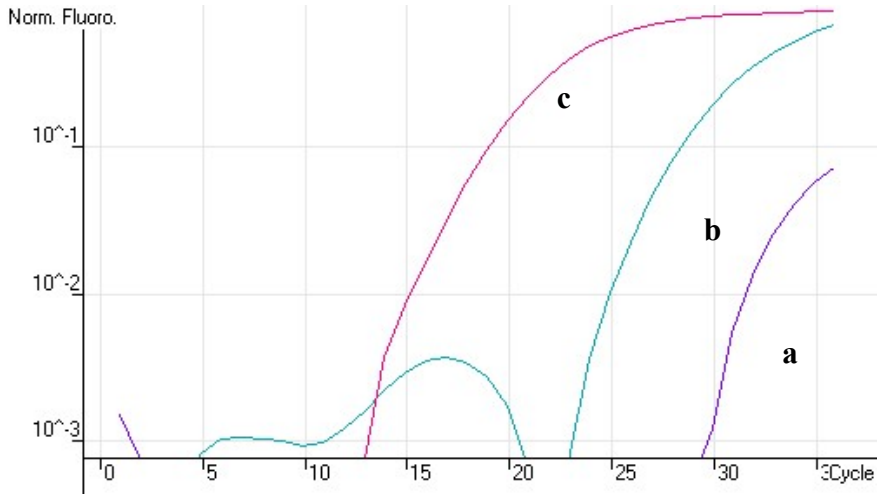

Figure 4.3: Verification of successful cDNA synthesis.
qRT-PCR curves for DNase treated RNA **(a);** RNA **(b)**; and cDNA **(c)** from the same biological sample.

In this project, the reference gene YWHAZ was used. Before analysis of target genes expression, YWHAZ was compared with another reference gene, β-actin across all

samples. The purpose was to confirm that YWHAZ expression was stable; the Δ-Ct value of the two reference genes was consistent throughout all samples, mean 4.57 ± 0.39 (Figure 4.4). This confirms the work of others, which suggests that YWHAZ is more stable in human endometrium.[205]

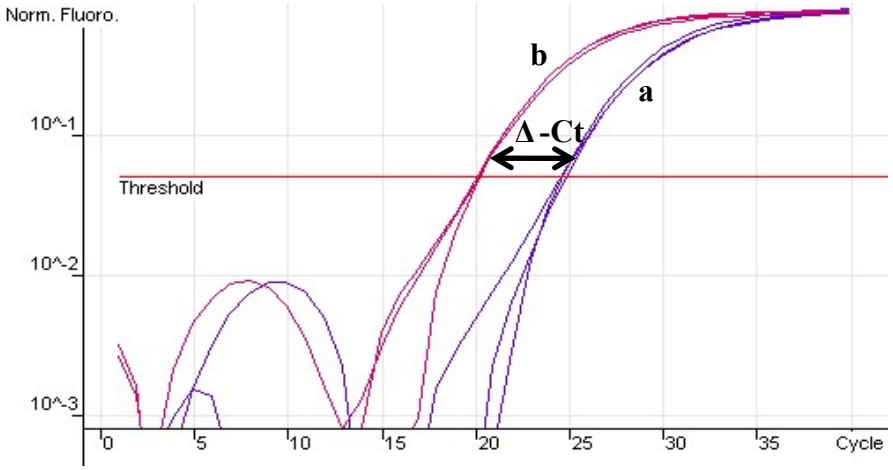

Figure 4.4: Verification YWHAZ stability
YWHAZ **(a)** and β-actin **(b)** gene expression was analysed. The Δ-Ct value was calculated and was constant across all cDNA samples**.**

Validation of primers was assessed via base pair (bp) size of qPCR products. Gel electrophoresis of qPCR products from control template was performed (Figure 4.5).

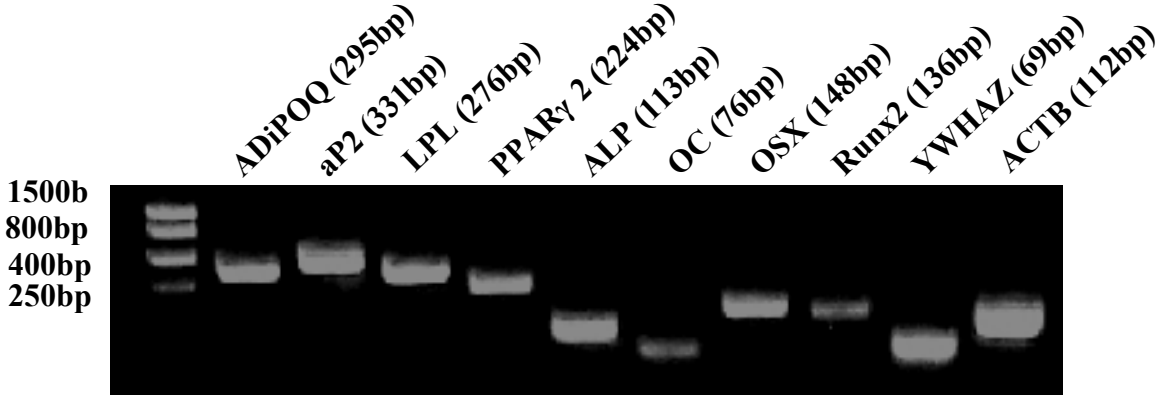

Figure 4.5. Verification of qPCR primers
Products of qPCR were run on a gel to ensure band size was correct.

Chapter Five: RESULTS

5.1: Assessment of unipotency of EEp-ASPCs *in vitro*

A total of eight patient samples were cultured in 3D media to produce gland-like structures, median age 30.5 years (range 27 – 47 years). Reasons for surgery included menorrhagia (without identified endometrial pathology, such as inflammation or malignancy), and non-endometrial related causes, such as sterilisation and cystectomy. Data are incomplete for three recruited patients (for patient demographics see Table 5.1). Gland-like structures were subjected to immuno-histochemical analysis and compared to reference tissue sections from three patients; eutopic endometrium from a woman suffering with endometriosis, a fertile control and a PM sample (for demographics of reference tissue see Table 5.2).

Table 5.1: *Patient demographics of samples used to assess unipotency of EEp-ASPCs*

No	Biopsy	Reason for Surgery	Endometriosis	Age	BMI	Gravida	Cycle Stage
1	FT	Menorrhagia	No	30	26.7	1	Proliferative
2	FT		No	47	27.7	4	Late Proliferative
3	Pipelle	Laparoscopy Dye Test	Yes	28	25.5	0	Mid-Secretory
4	Pipelle	Cystectomy	Yes	39	24.8	0	Late Proliferative
5	Pipelle	Diagnostic Laparoscopy	Yes	21	18.7	1	Proliferative
6	Pipelle		No	38	26.6	2	Menstrual
7	Pipelle	Laparoscopic Sterilisation	No	27	23	5	Early Secretory
8	Pipelle		No	27	21.1	4	Secretory

Table 5.2: *Patient demographics for samples used as reference tissue for gland-like structures*

Reference	Biopsy	Reason for Surgery	Age	BMI	Gravida	Cycle Stage
Endometriosis	FT	Endometriosis	45	20.5	2	WOI
Fertile Control	FT	Pelvic Pain NAD	30	28.1	3	WOI
PM	FT	Prolapse	77	26.6	2	N/A

5.1.1: EEp-ASPCs are capable of producing gland-like structures in 3D culture

After 14 days in 3D culture, all samples generated single lumen spheroids/organoids/gland-like structures. Two types existed; comprised of non-polarised cells, and those comprised of polarised cells, with the former accounting for approximately 90%. It is the polarised structures that are the subject for much of this study. No difference in morphology was observed between samples taken from endometriosis patients and fertile controls, other than the general observation that endometriosis samples produced spheroids/organoids at faster rate. 3D structures morphologically mimicked glands present in human endometrium. Cells showed positive immuno-reactivity for the epithelial marker CK18, and adhesion molecule β-catenin, confirming that cells structures were epithelial (Figure 5.1). *pan*Actin at the apical surface of the epithelia illustrated that cells were polarised and differentiated. The *pan*Actin staining pattern differed between *ex vivo* and *in vivo* glands; being more localised in endometrial tissue. Interestingly, one *ex vivo* gland demonstrated the classical *in vivo* appearance on half of the gland, whilst maintaining the *ex vivo* pattern on the other (Figure 5.1f).

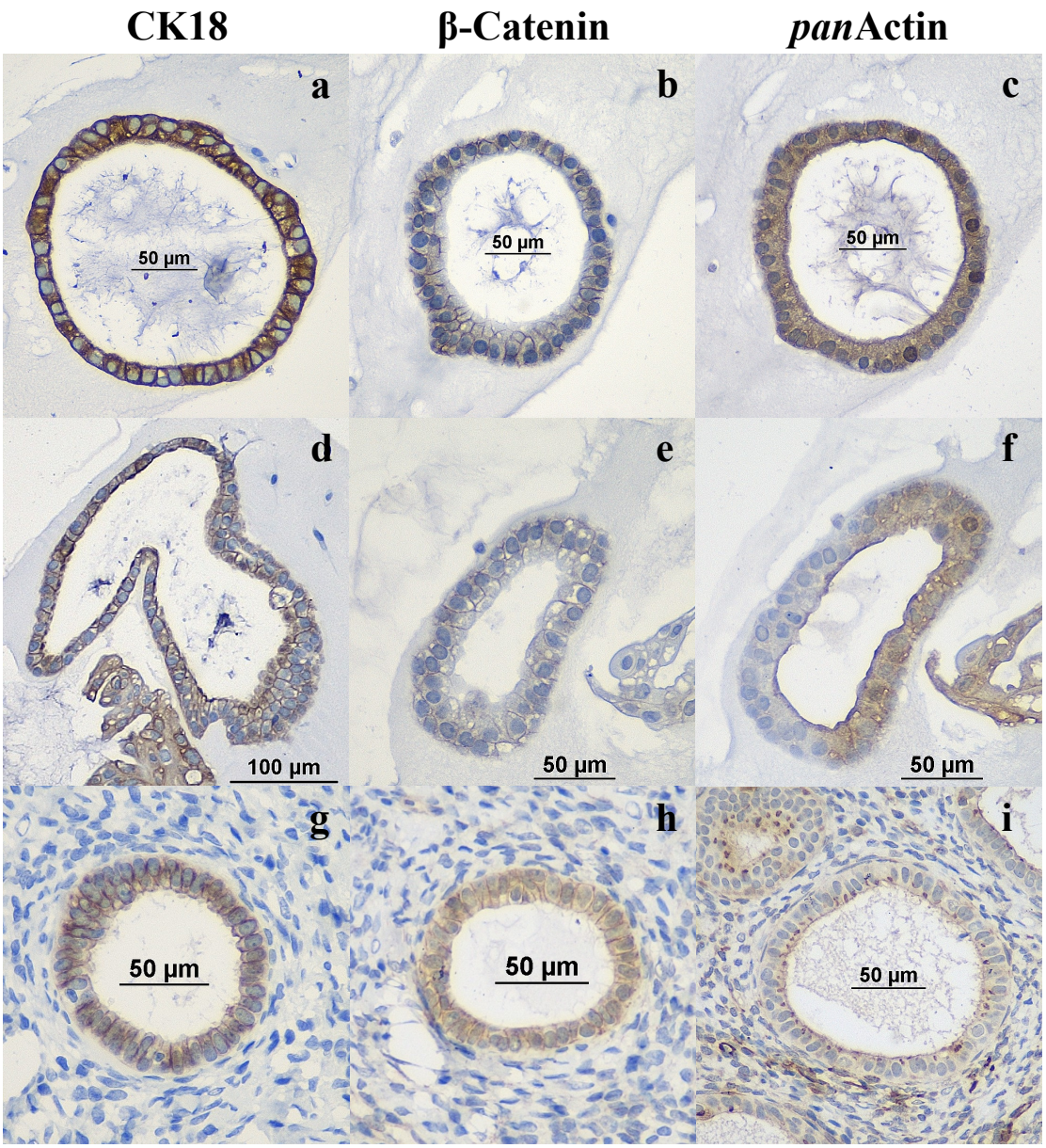

Figure 5.1: EEp-ASPCs are capable of producing gland-like structures in 3D culture
After 14 days in 3D culture, gland-like structures developed **(a-f)**. These resembled glands formed in endometrial tissue sections **(g-i)** and stained positive for structural proteins CK18 **(a,d,g)**; β-Catenin **(b,e,h)** and *pan*Actin **(c,f,i)**.

5.1.2: Gland-like structures best resemble post-menopausal glands from endometrial tissue

Polarised gland-like structures were mainly spherical or elliptical in shape and resembled either basal or PM endometrial glands. They did not resemble the highly coiled and differentiated glands of functional endometrium seen at the progesterone-dominant WOI (Figure 5.2). Steroid receptor expression profile of the oestrogen receptor subtypes ER-α, ER-β and PR was compared across the five groups, and data demonstrated that organoids best resemble PM endometrial glands for the following reasons.

- Expression of ER-α in reference glands was almost negligible in the basal layer, and greater in the functional layers of both groups of cycling endometrium. The intensity and number of positive cells was maximal in PM endometrium. Mean expression of ER-α in gland-like structures was 88.62 ± 8.74% compared with 81.70 ± 10.17% in PM tissue.
- A similar effect was observed with ER-β; more staining was visible in the functionalis when compared with the basal layer, and strongest in PM endometrium. ER-β expression was 94.47 ± 5.55% in gland-like structures versus 99.71 ± 0.93% in PM endometrium.
- PR expression in reference glands was intense in both layers of all cycling endometrium. PR was expressed at a lower extent in PM endometrium, and even less so in gland-like structures; 83.79 ± 9.06% versus 31.79 ± 9.31% respectively.

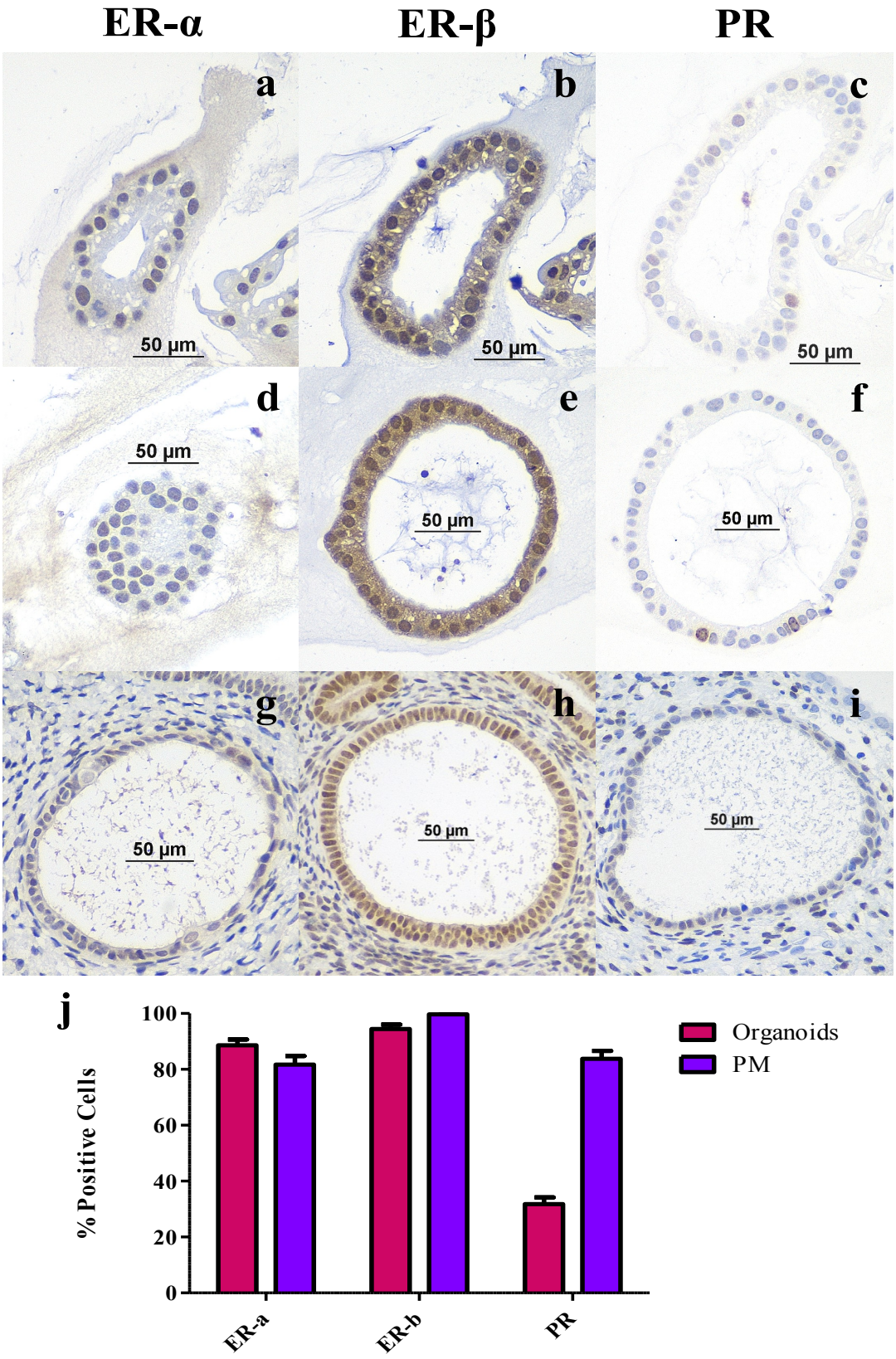

Figure 5.2: Gland-like structures best resemble post-menopausal glands from endometrial tissue. Organoids **(a-f)** were compared with human pre- and post-menopausal endometrium **(g-h)**. ER-α, ER-β and PR was expressed in all structures. However, based on hormonal receptor profile, gland-like structures best resembled PM glands **(j)**.

5.1.3: Gland-like structures demonstrate negative immuno-reactivity for ESC transcription factors

Cells comprising the gland-like structures demonstrated negative immuno-reactivity for markers of an undifferentiated state; NANOG, OCT4a and SOX2. Some NANOG and OCT4a staining was observed in most cells. However, upon colour de-convolution, it became clear the staining was not nuclear and thus considered to be negative (Figure 5.3).

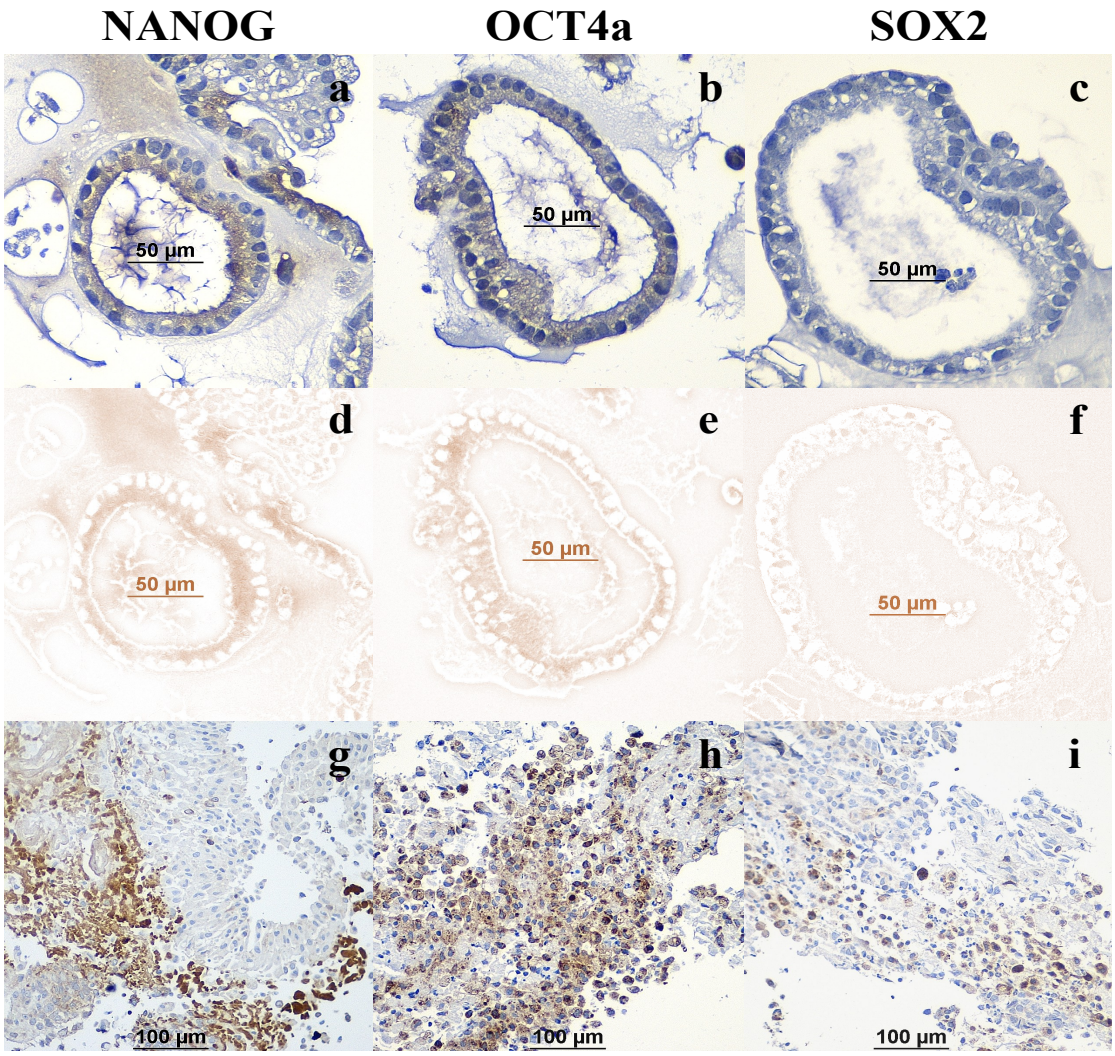

Figure 5.3: Gland-like structures demonstrate negative immuno-reactivity for ESC transcription factors. Gland-like structures showed negative immuno-reactivity for undifferentiated markers NANOG **(a)**; OCT4a **(b)** and SOX2 **(c)**. NANOG and OCT4a produced some staining, however, upon colour de-convolution **(d & e respectively)**, staining was not nuclear. Control tissue was endometrial cancer, which had nuclear and non-nuclear staining for NANOG **(g)**; OCT4a **(h)** and SOX2 **(i)**.

5.1.4: Gland-like structures show positive immuno-reactivity for ASPC markers

Gland-like structures were stained with the ASPC markers PODXL, CK5/6 and SSEA-1.

- PODXL staining in reference tissue was present in vascular endothelia and upon the apical surface of endometrial glands. Epithelial staining was greatest in PM tissue. Staining of gland-like structures revealed cells had less immuno-reactivity for PODXL when compared to reference tissue (Figure 5.4).
- SSEA-1 was strongly expressed, and present on the apical surface of cells in all reference groups. SSEA-1 staining intensity was strongest in PM tissue and the basal layers of both endometriosis and fertile control endometrium. All cells comprising polarised gland-like structures showed strong intensity staining for SSEA-1 (Figure 5.4).
- In reference tissue, CK5/6 stained epithelia only; no CK5/6+ stroma or endothelia were observed. Luminal epithelia were stained consistently; several glands were stained positive for CK5/6 (Figure 5.4). Interestingly, undefined structures consisting of cells that did not express CK18 were present in the 3D culture from patient 5, which showed strong immuno-reactivity for CK5/6. (Figure 5.6).

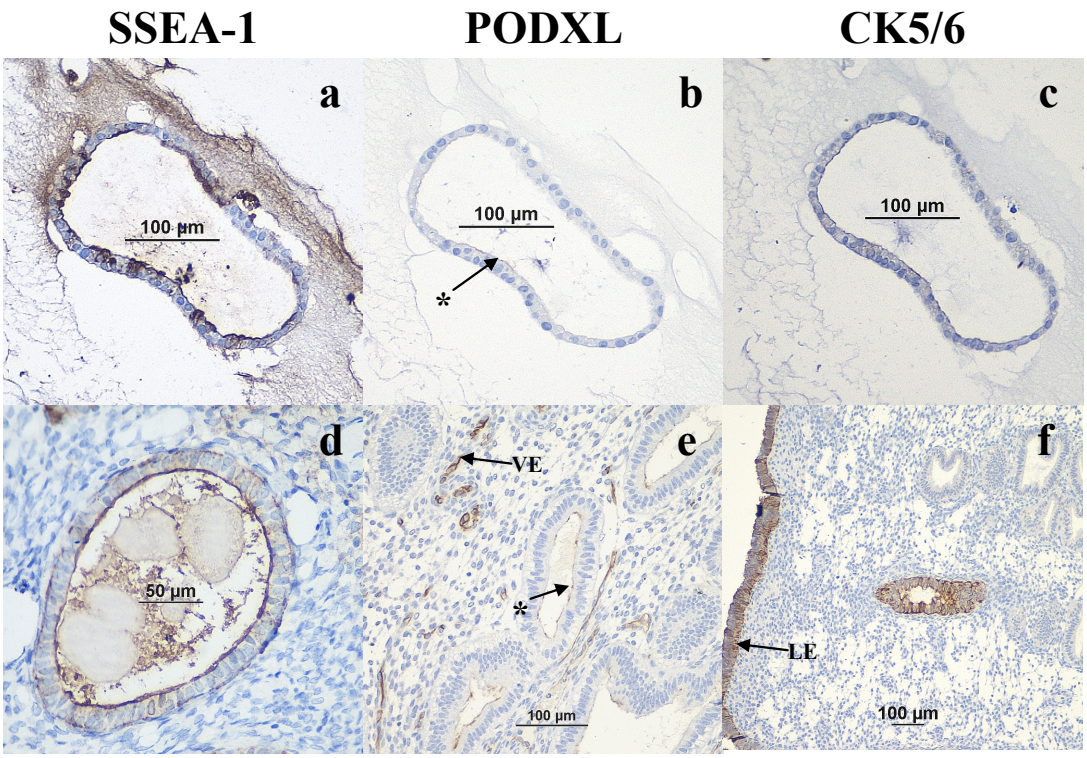

Figure 5.4: Gland-like structures show positive immuno-reactivity for ASPC markers. Gland-like structures demonstrated strong immuno-reactivity for SSEA-1 **(a)**, similar to glands in PM and basal tissue **(d)**. PODXL was present on the apical surface of some gland-like structures, see *** (b)**, but was considerably less intense than seen in tissue **(e)**, see *****, where gland and vascular endothelia were PODXL+, see **VE**. CK5/6 was not seen in gland-like structures **(c)**, and in reference tissue **(f)** luminal epithelia were consistently stained, see **LE**, along with occasional whole glands.

5.1.5: Expression of markers of cell fate in gland-like structures showed that cells are actively proliferating

As discussed earlier, gland-like structures were analysed for immuno-reactivity to markers of proliferation, senescence, and apoptosis such as Ki67, telomerase, 8-OHdG, H_2AX, M30 and CPP32 to determine cell fate. Several Ki67+ cells were present in each gland-like structure indicating proliferation. Organoids had a strong staining intensity for telomerase, suggestive of progenitor features. Senescent makers 8-OHdG and H_2AX were present in certain cells of each organoid. Unfortunately, it

was difficult to establish sequential sections for these antibodies. Therefore, it cannot be determined whether some cells had strong intensity for Ki67 yet negative for oxidative stress and senescent markers 8-OHdG and H_2AX, which would be indicative of an ASPC. Nonetheless, all cells in organised structures were negative for apoptotic markers CPP32 and M30. A very small number of cells in non-specific clusters were M30+ (Figure 5. 5g).

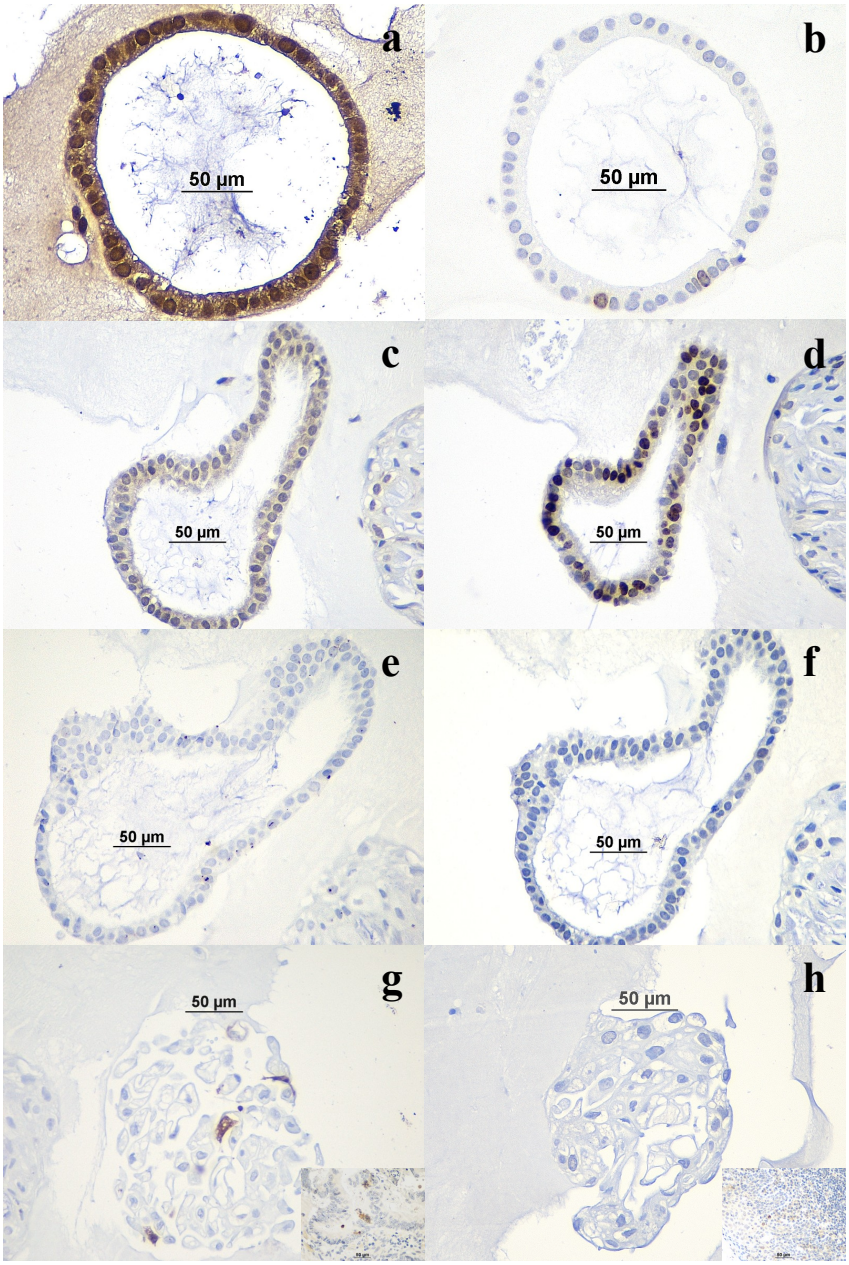

Figure 5.5: Gland-like structures are actively proliferating
Gland-like structures demonstrated positive immuno-reactivity for telomerase **(a)**, H_2AX **(b)**, 8-OHdG **(c)** and Ki67 **(d)**. Apoptotic markers M30 and CPP32 were negative in gland-like structures **(e & f respectively)**. M30 was present in some cells that were aggregated in loose clusters **(g)**, but CPP32 was not **(h)**. Insets represent controls.

5.1.6: 3D culture supports growth of non-gland-like structures

3D structures generated from patient 5, harvested at day 10 and 14, produced non-gland-like, as well as gland-like structures. Morphologically, these structures resembled distinct solid spheres, unlike anything observed in reference tissue. Solid structures appeared to comprise epithelia on the outer circumference, which stained positive for CK18, β-Catenin and *pan*Actin, and an inner mass, which did not express any of these structural proteins (Figure 5.6). The inner mass of cells was negative for apoptotic markers CPP32 and M30. Interestingly, these cells were also negative for the steroid receptors, and markers of proliferation and senescence. Likewise, cells were also negative for ESC makers and APSC markers PODXL and SSEA-1. Out of all the markers used in this study, cells comprising the inner mass only showed positive immuno-reactivity to CK5/6.

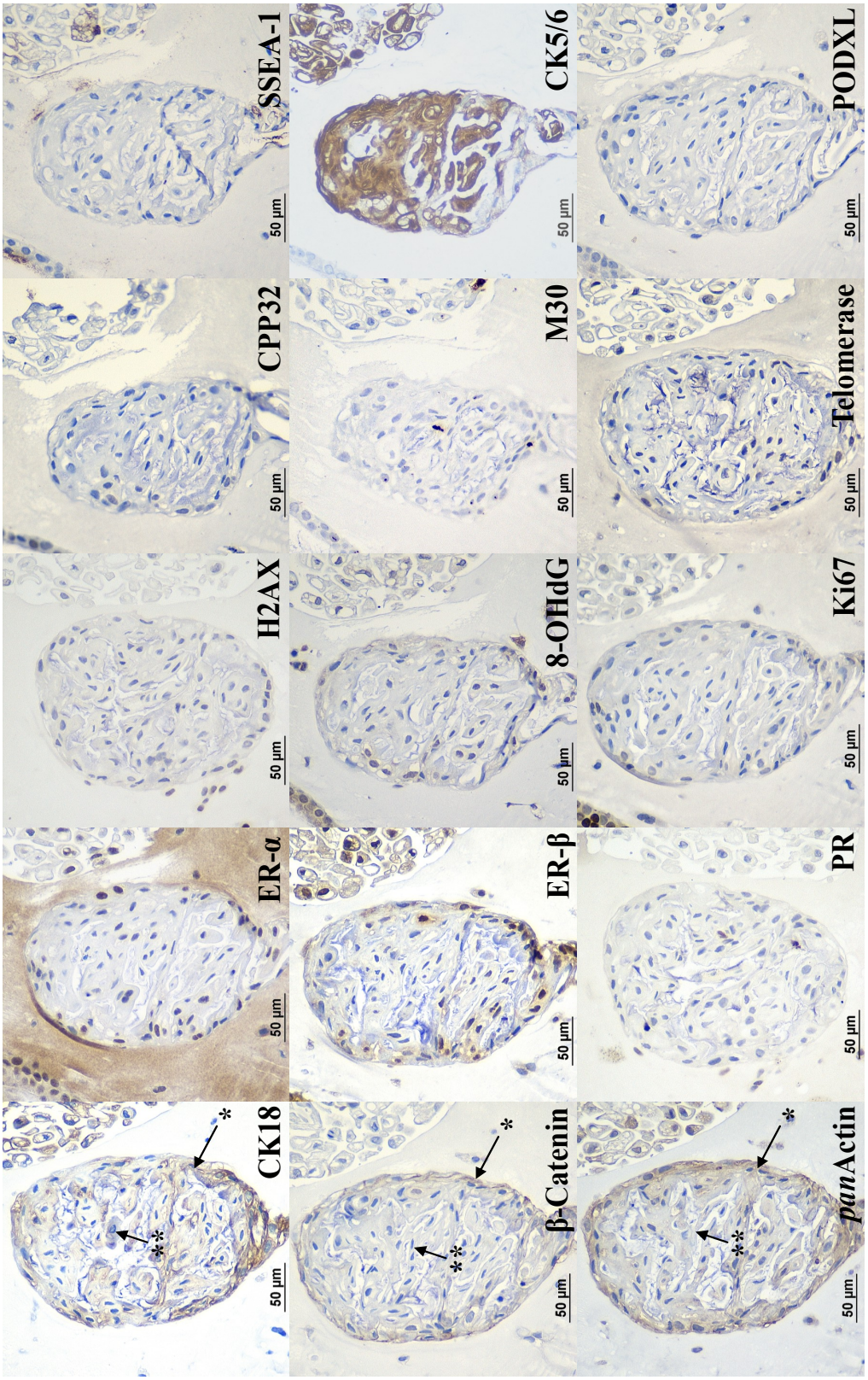

Figure 5.6: 3D culture supports growth of non-gland-like structures. Solid spheres formed in 3D culture that did not resemble gland-like structures. These appeared to consist of two cell types – an outer layer that resembled epithelia, staining positive for the structural proteins CK18, β-Catenin and *pan*Actin **(*)**, and an inner mass that were negative **(**)**. Inner mass cells were negative for ER-α, ER-β and PR. Cells also were negative for H_2AX, 8-OHdG and apoptotic markers CPP32 and M30. Telomerase, Ki67, SSEA-1 and PODXL were negative, but interestingly, CK5/6 was present in all cells within the inner mass.

5.2: Assessment of multipotency of EEp-ASPCs *in vitro*

Samples from eleven patients were collected and cultured in either adipogenic or osteogenic media, to promote mesodermal differentiation. The median age of these patients was 45 (range 31 to 47 years; for patient demographics see Table 5.3). Cells from sample 19 did not survive in either the control or interventional media. All remaining samples were stained at the end of the 14-day protocol. The 3 samples with the highest set of mRNA concentration and 260/280 values were assessed for gene expression: samples 12, 14 and 15 for the adipogenic assay and 9, 12 and 13 for the osteogenic assay (for mRNA concentration and 260/280 values see Table 5.4). hMSC were cultured in the same conditions and acted as a positive control.

Table 5.3: Patient demographics of samples used to assess multipotency of mesodermal lineage

No.	Biopsy	Reason for Surgery	Endometriosis	Age	BMI	Gravida	Cycle Stage
9	FT	Menorrhagia	No	45	33.1	2	Proliferative
10	FT	Fibroids	No	46	26.8	6	Late Secretory
11	FT	Fibroids	No	47	23.4	2	Early Proliferative
12	FT	Menorrhagia	No	46	23.1	3	Late Proliferative
13	FT	Menorrhagia	No	46	27.4	2	Secretory
14	FT	Menorrhagia	No	31	24.9	3	Late Secretory
15	FT	Prolapse/Fibroid	No	47	21.6	2	Proliferative
16	Pipelle	Endometrioma	Yes	31	28.3	1	WOI
17	Pipelle	Menorrhagia	Yes	31	26.8	2	Secretory
18	Pipelle		No	38	28.1	3	WOI
19	Pipelle	Menorrhagia	No	32	52.2	1	Proliferative

Table 5.4: mRNA concentration and 260/280 values for samples used to assess multipotency of EEp-ASPCs

	mRNA concentration (ng/□l)						260/280 ratio					
	A+	A-	O+	O-	C+	C-	A+	A-	O+	O-	C+	C-
9	**167.6**	**139.1**	**1195.1**	**732.1**	**655.5**	**1749.0**	**1.79**	**1.69**	**1.93**	**1.91**	**1.94**	**1.95**
10	131.7	117.7	-	-	6.8	119.8	1.69	1.85	-	-	1.40	1.78
11	145.4	158.9	52.6	245.3	75.7	143.9	1.84	1.85	1.93	1.92	1.92	1.77
12	**117.7**	**172.0**	**385.5**	**473.6**	**517.6**	**424.2**	**1.68**	**1.85**	**1.90**	**1.90**	**1.90**	**1.90**
13	**99.6**	**267.1**	**360.8**	**563.9**	**438.8**	**200.0**	**1.77**	**1.89**	**1.82**	**1.93**	**1.93**	**1.76**
14	**172.8**	**277.7**	**-**	**-**	**256.4**	**683.2**	**1.86**	**1.87**	**-**	**-**	**1.91**	**1.92**
15	**130.0**	**150.7**	**-**	**-**	**133.3**	**319.4**	**1.81**	**1.86**	**-**	**-**	**1.85**	**1.91**
16	62.0	113.6	429.6	512.8	1159.1	658.6	1.68	1.77	1.95	1.95	1.95	1.91
17	94.3	126.7	191.9	777.4	313.8	475.0	1.83	1.76	1.84	1.89	1.88	1.92
18	-	-	125.0	186.9	173.1	217.4	-	-	1.81	1.57	1.62	1.85
19	-	-	-	-	-	-	-	-	-	-	-	-

5.2.1: hMSCs are multipotent

In order to verify that our media promoted differentiation of ASPCs into other cell types, hMSCs were cultured and served as a positive control. After two weeks of culture in appropriate media, hMSCs were able to differentiate into adipocytes and osteoblasts.

5.2.1i: Adipogenesis

In adipogenic differentiation media, hMSCs began to show changes in appearance after only 3 days in culture; cells became round and accumulated lipid vacuoles by day 14, which stained positive for Oil Red O. Cells cultured in control media did not produce the same morphology (Figure 5.7). mRNA analysis also confirmed

successful differentiation; PPARγ2 and LPL, both early markers of adipogenic differentiation, were significantly increased by a mean factor of 29.1 (p=0.02) and 3468.3 (p=0.0001) respectively, when compared to cells cultured in control conditions. Similarly, a more mature marker, aP2 increased by a mean factor of 18011.9 (p= 0.0001). Although ADiPOQ expression also increased by a factor of 1332.6, this apparent increase was not found to be statistically significant.

5.2.1i. Osteogenesis

In osteogenic differentiation media, cells became more spiculated and stained positive for ALP after 14 days in culture (Figure 5.8). Again, osteogenic differentiation of hMSC was further confirmed by qPCR. Early markers ALP and OSX were significantly increased by a mean factor of 5.1 (p=0.001) and 6.9 (p=0.0001) respectively. Early osteogenic marker Runx2 was increased by a factor of 1.7, yet this was found to be statistically insignificant. Interestingly, OC was down-regulated by a mean factor of 0.5 without this apparent decrease in gene expression reaching statistical significance.

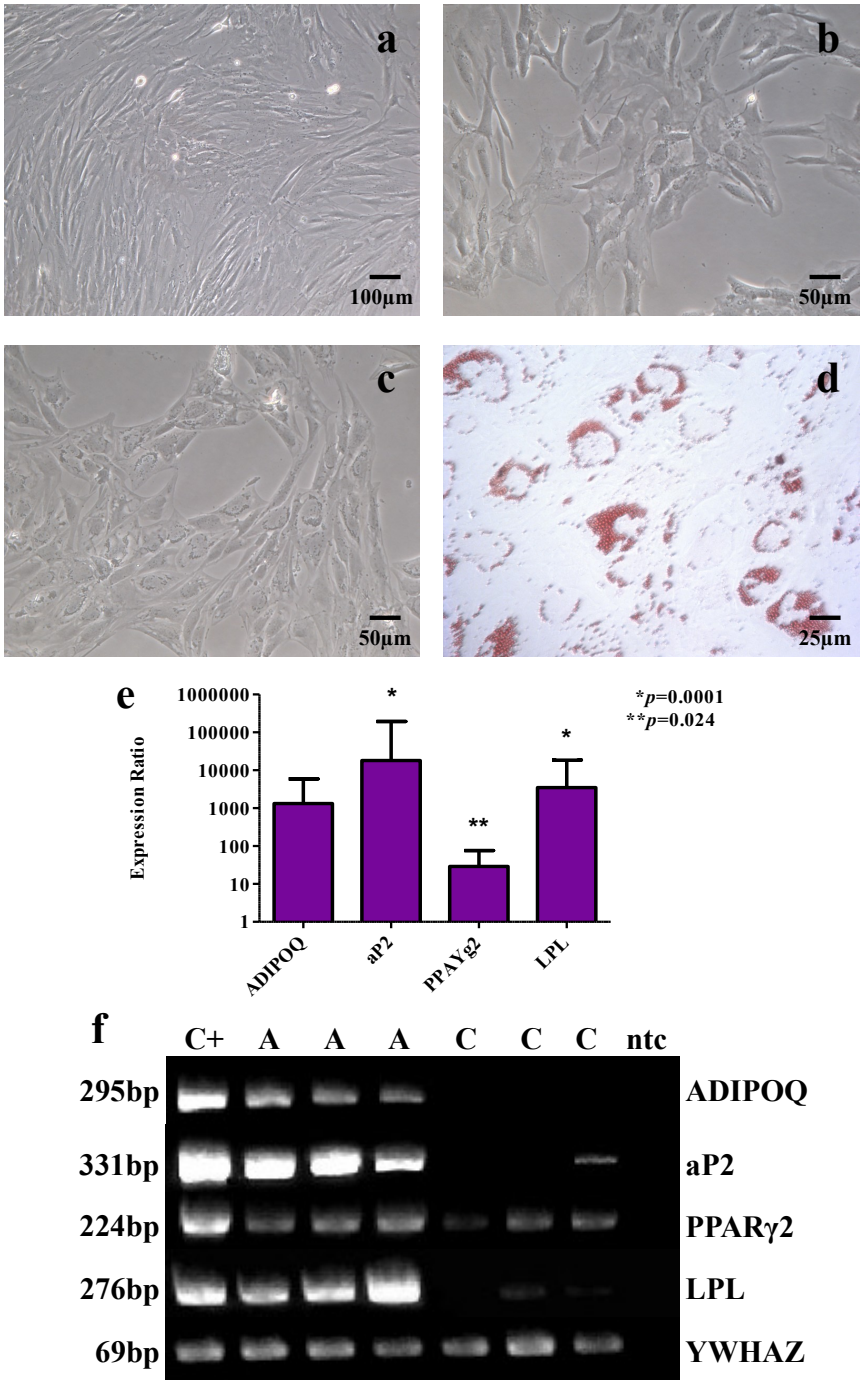

Figure 5.7: hMSCs are capable of adipogenesis
Microscopic images show hMSCs in adipogenic media at day 1 **(a)**, day 5 **(b)**, day 10 **(c)**, and Oil Red O staining on day 15 **(d)**. aP2, PPARγ2 and LPL mRNA were up-regulated in adipogenic-induced hMSCs **(e)**. Gel electrophoresis of products **(f)**, A – adipogenic hMSC, C- control media hMSC.

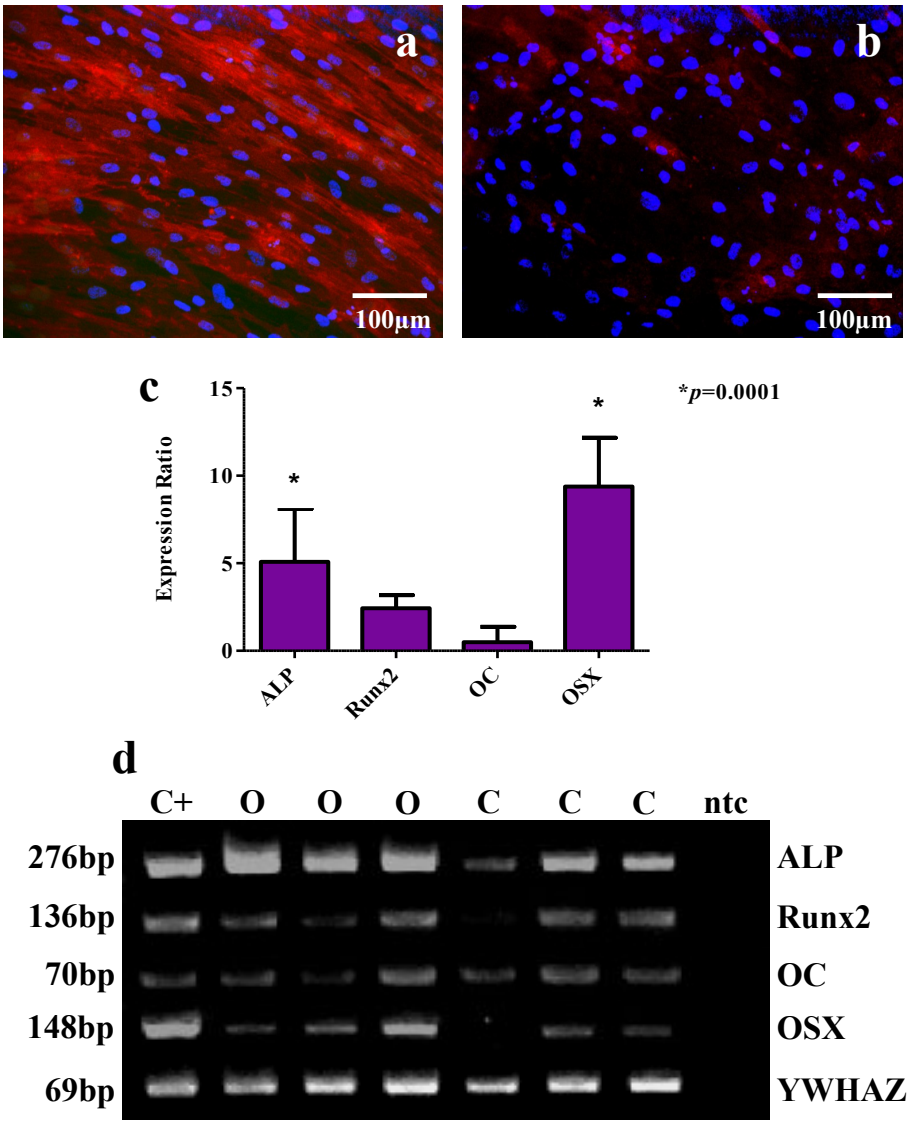

Figure 5.8: hMSCs are capable of osteogenesis.
Fluorescent images of ALP staining of hMSCs after 14 days in osteogenic **(a)** and control **(b)** media. Expression of ALP and OSX were significantly increased **(c)**. Gel electrophoresis of products **(d)**, O – osteogenic hMSC, C-control media hMSC.

5.2.2: EEp-ASPCs are not multipotent

5.2.2i. Adipogenesis

Both SSEA-1 enriched and depleted populations began to 'round up' after one week in adipogenic media, which was not observed in the control. It was also noted that cells in adipogenic media became sparser each day. Again, this did not happen in the control, suggesting that the 'rounding up' is not representative of adipogenic differentiation but cell senescence. Consequently, staining with Oil Red O was negative with one minor exception (Figure 5.9). An individual cell in the SSEA-1- fraction produced small vacuoles. Admittedly, these vacuoles were considerably smaller than those observed in induced hMSCs (Figure 5.7). Some tiny Oil Red O vacuoles were also present in the control of both fractions. mRNA expression of early markers PPARγ2 and LPL in three samples were analysed to confirm findings. There was no statistical difference of PPARγ2 expression of SSEA-1+ cells. Conversely, in the SSEA-1- fraction, PPARγ2 expression increased by a mean factor of 2.6 (p=0.0001). Expression of LPL did not change significantly in either the SSEA-1+ or SSEA-1- fraction.

5.2.2ii. Osteogenesis

Cells in both SSEA-1+ and SSEA-1- populations did not change morphology over the period of investigation. However, it also was noted that endometrial epithelial cells thrived in the osteogenic media, and this was particularly true for contaminating stromal cells. When RNA was quantified, it was also observed that total RNA concentrations were considerably higher for cells that had been cultured in the osteogenic media (Table 5.4). A very small number of cells stained positively for ALP in both the SSEA-1+ and SSEA-1- fractions (Figure 5.10) but this was also

similar with cells in control media. qPCR confirmed there was no change in expression of osteoblast markers ALP or OSX in the SSEA-1+ fraction. Interestingly, in the SSEA-1-population OSX actually decreased in the sample group by a mean factor of 0.092 (p=0.0001).

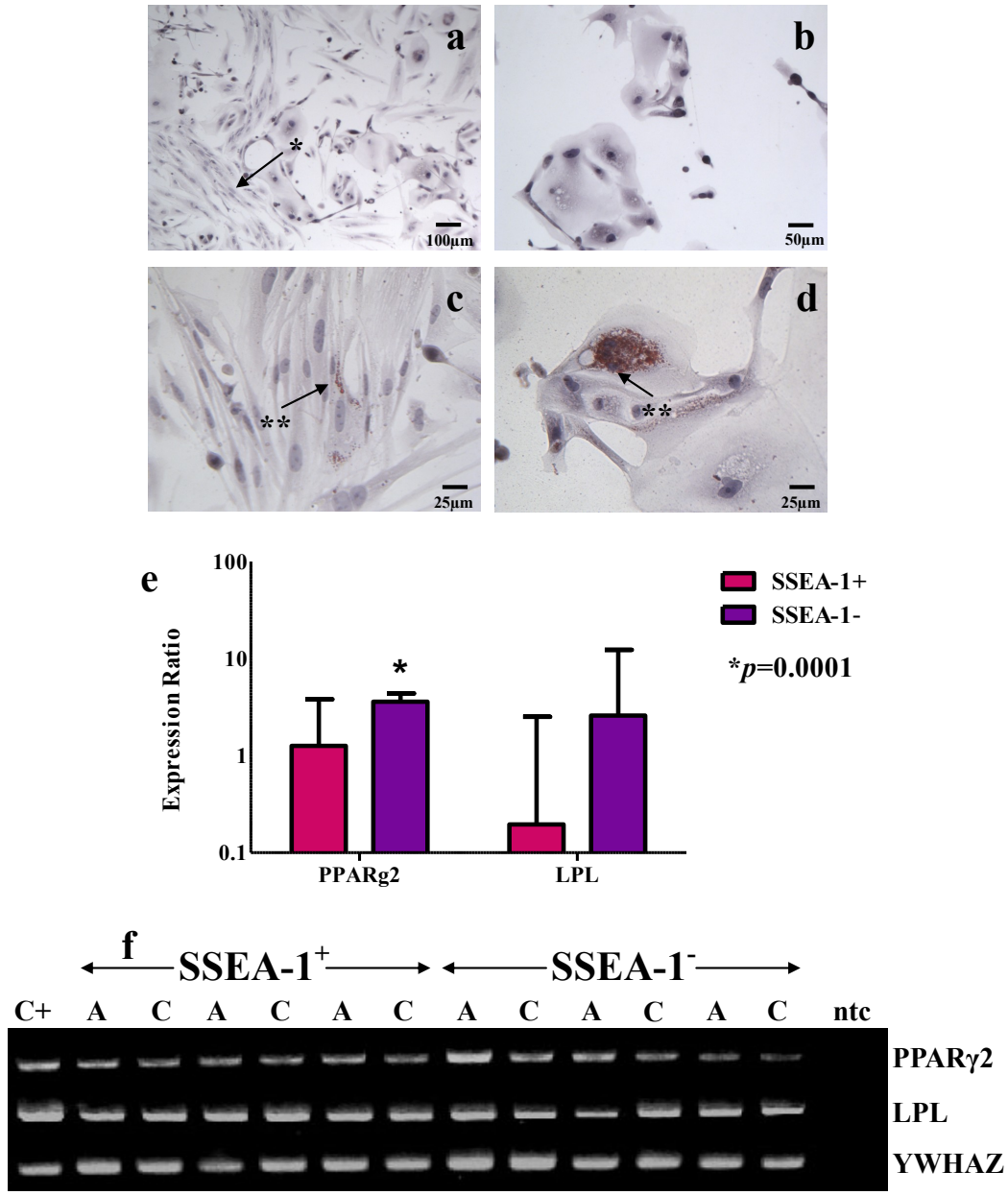

Figure 5.9: EEp-ASPCs are unable to undergo adipogenesis
SSEA-1 depleted populations had a higher degree of stromal contamination **(a)**, see *. Epithelial cells grew in small clusters and SSEA-1+ cells depleted in number throughout the time of the experiment **(b)**. One cell in the SSEA-1 depleted fraction produced lipid vacuoles, which stained positive for Oil Red O **(c)**, see **. Cells cultured in control media also produced tiny lipid vacuoles **(d)**, **. Gene expression analysis demonstrated levels of PPARγ2 and LPL remained unchanged in the SSEA-1 enriched fraction. However, PPARγ2 expression increased in the SSEA-1 depleted population **(e)**. Gel electrophoresis demonstrates products of qPCR **(f)**, A- cells in adipogenic medium, C- cells in control medium.

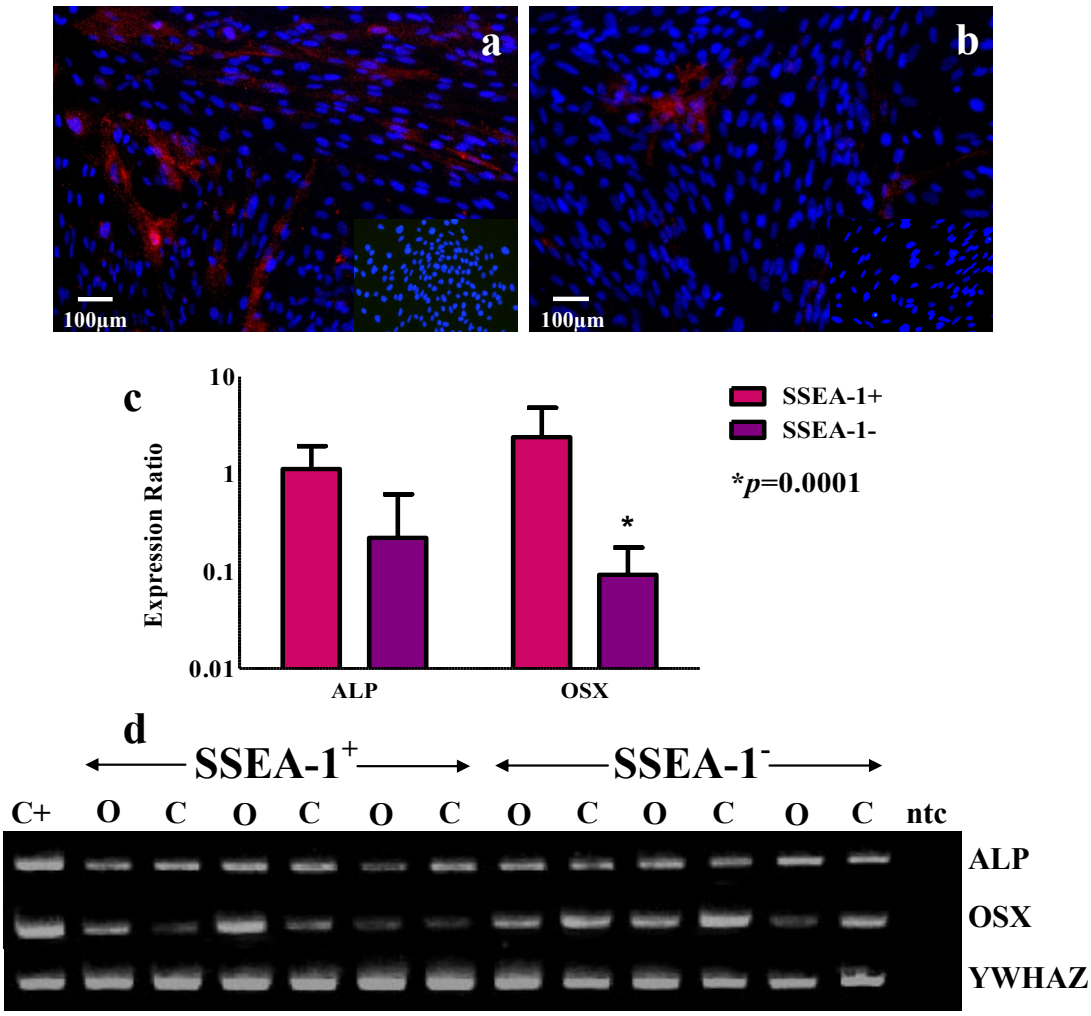

Figure 5.10: EEp-ASPCs are unable to undergo osteogenesis
Fluorescent images of ALP staining of SSEA-1 enriched **(a)** and depleted **(b)** populations after 14 days in osteogenic media. There was no change in ALP expression during the assay, however, OSX was actually down-regulated in the SSEA-1- fraction **(c)**. Gel electrophoresis of products **(d)**, O – osteogenic induced cells, C- control media hMSC.

5.3: Assessment of pluripotency of EEp-ASPCs *in vitro*

Three patients were recruited for the study of neurogenic trans-differentiation (for demographic data see Table 5.5). SSEA-1+ and SSEA-1- endometrial epithelial cells were cultivated in neurogenic media, and analysed with PGP9.5, a mature pan-neuronal marker after 10 days (Figure 5.11). Cells cultured in epithelial media served as a control. Cells in both SSEA-1+ and SSEA-1- fractions became more elongated in the neurogenic media. An increased mean percentage of PGP9.5 positive cells were observed in cells cultured in the neurogenic media, when compared to those in the control; 27.1 ± 6.9% vs.11.4% ± 2.0% respectively (p=0.03).

Figure 5.5: Patient demographics for trans-differentiation assay

No.	Biopsy	Reason for Surgery	Endometriosis	Age	BMI	Gravida	Cycle Stage
14	FT	Menorrhagia	No	31	24.9	3	Late Secretory
20	FT	Mittelschmerz	No	44	38	4	Early Proliferative
21	FT	Fibroids	No	44	31.6	3	Early Proliferative

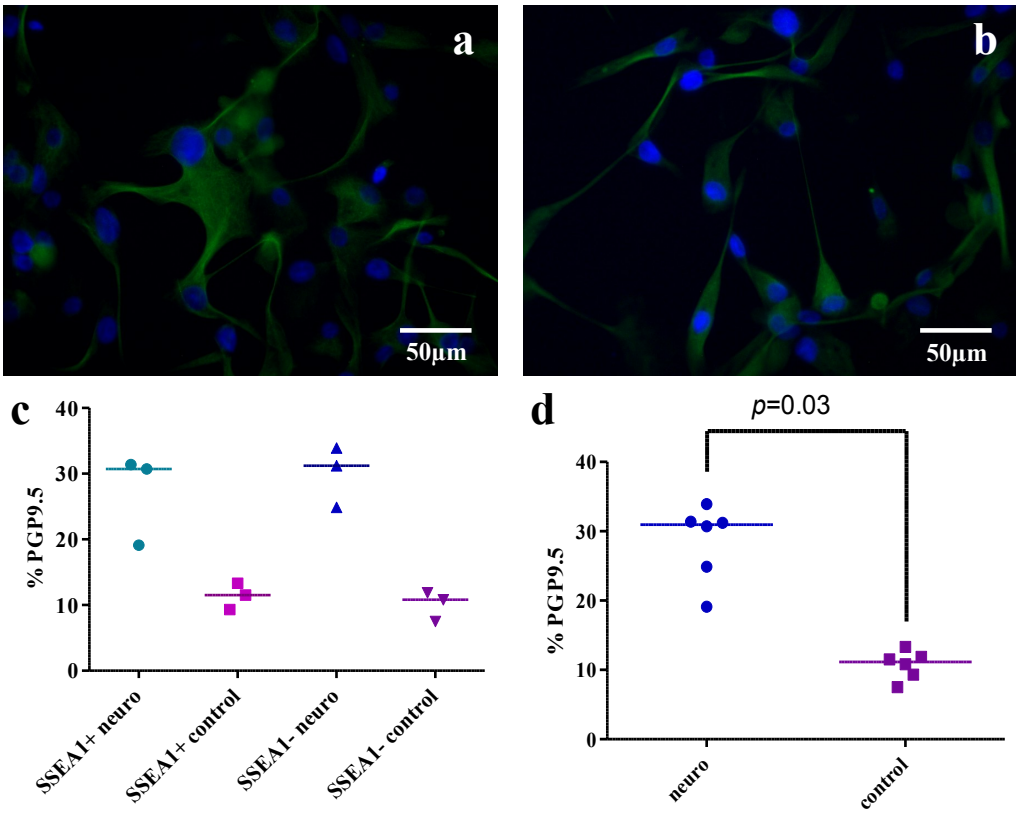

Figure 5.11: Assessment of pluripotency of EEp-ASPCs in vitro
The percentage of PGP9.5 positive cells increased in SSEA-1 enriched **(a)** and depleted **(b)** fractions cultured in neurogenic media when compared with controls **(c)**. However, this was only significant when the two populations were combined **(d)**.

Chapter Six: DISCUSSION

This was an *in vitro* study exploring the differentiation potential of endometrial SSEA-1+ and SSEA-1- epithelial cells. The primary aim was to verify unipotency. This required the use of a previously developed 3D culture model to form gland-like structures/organoids. A panel of 18 markers was used to analyse the organoids and validate their resemblance to *in vivo* tissue. Differentiation into other lineages was also attempted, which encompassed the primary cell culture of SSEA-1+ and SSEA-1- cells, in media that promoted both mesodermal and ectodermal differentiation.

6.1: SSEA-1+ cells have a greater propensity to differentiate into endometrial glands

Gland-like structures formed within 14 days of 3D culture; consistent with work by *Fritja et al*., in mouse endometrium.[206] Organoids demonstrated polarity via the presence of apical actin and basal nuclei. Based on immuno-histochemical analysis, organoids had positive immuno-reactivity for the hormones implicated in the menstrual cycle. Together, these findings suggest the structures formed are representative of endometrial glands *in vivo,* and thus we can validate our 3D model. A low number of apoptotic cells and high number of Ki67+ cells supports that our model is a suitable environment to study endometrial epithelial behaviour. This is coherent with other work, which implies cells behave similarly in a 3D environment to how they would *in vivo.*[207]

Recently, it was acknowledged that SSEA-1+ cells had a greater propensity to produce gland-like structures. Similarly, SPCs from the intestine are capable of

reproducing tissue specific structures within a 3D environment.[208] Therefore, we further support our previous work that SSEA-1 is an EEp-ASPC marker by confirming that *in vitro* tissue reconstitution has been successful. It is likely these differentiated structures are a consequence of stem cell activity, as markers of an undifferentiated state; NANOG, OCT4a, SOX2 are present in cells in 2D culture but lost when transferred to a 3D environment (data not shown).

6.2: Gland-like structures best resemble in vivo glands in PM tissue

Human endometrium is a dynamic organ that undergoes monthly remodelling through differentiation and shedding. Functional glands at the WOI are regarded as the most differentiated epithelia; the basalis and PM tissue are thought to harbour the SC niche, with germinal glands to produce the transient functionalis. We compared gland-like structures, formed in 3D culture, with human endometrial glands *in vivo* (tissue sections), to assess 'how differentiated' the 3D structures actually were. Polarised organoids were spherical and elliptical in shape; they did not reproduce the tortuous and highly coiled structures associated with glands at WOI. In fact, structures were very similar in size and morphology to basal and PM glands. Furthermore, based upon steroid expression, organoids best resembled PM glands on account of low PR expression. These findings could be explained by two theories:

1. Gland-like structures could be representative of a progenitor population. PM and basal glands are thought to harbour the SPC niche: it is this layer of endometrium only that is retained during menstruation and the menopause.[173] The idea is supported by work by *Prianishnikov*, where PR expression is believed to be acquired at terminal differentiation and absent on SPCs.[124] Although apical actin was present on structures, this was less localised than in *in vivo* tissue, suggesting that gland-like structures are not

completely differentiated. In addition, it is interesting that the cells in this assay were not sorted on SSEA-1, yet gland-like structures demonstrated positive immuno-reactivity for the glycan.

2. More likely, gland-like structures best resemble PM tissue on account of the similar inert hormonal environment. Within pre-menopausal tissue there are cyclical changes in oestrogen and progesterone, affecting the morphology and function of endometrial cells; oestrogen encourages growth and proliferation, whereas progesterone opposes it. It could be considered surprising for endometrial gland-like structures to proliferate in an environment without oestrogen supplementation. However, our findings echo the work of *Bukovsky et al.,* which demonstrated that ovarian epithelia are capable of oestrogen dependent oogenesis *in vitro* without the need of exogenous oestrogen.[209] Successive work has demonstrated that the culture media DMEM/F12 may be concealing a hidden source of oestrogen. Phenolsulfanphthalein, the Phenol Red compound, used as a pH indicator in most culture medium, contains a lipid impurity, which has a 50% affinity to the oestrogen receptor when compared to oestradiol.[210] The presence of this compound could well explain why organoids have been capable of proliferation and partial differentiation in our 3D model. The same compound has been found to have a 0.2% potency when compared to oestradiol, and this low figure could justify why gland-like structures do not resemble the fully differentiated glands seen in human endometrium at WOI, when oestrogen is high.[210] Out of the *in vivo* reference groups, PM endometrium is exposed to the lowest levels of circulating oestrogen and best represents our 3D model.[211] Similarly, PM endometrium is also exposed

to the lowest levels of progesterone. PR expression levels in organoids were most similar to PM tissue. Currently, there is no data to suggest DMEM/F12 contains any progesterone-like compounds and again supports our findings.

6.3: EEp-ASPCs are unipotent

Our findings suggest that SSEA-1+ SPCs are unipotent. We have demonstrated that SSEA-1+ cells are representative of an SPC population by demonstrating the ability of *in vitro* tissue re-constitution. However, when the same cells were subjected to multi-lineage differentiation, the results demonstrated SSEA-1+ cells were unable to differentiate into adipocytes and osteoblasts. It is likely that SSEA-1 is representative of progenitor TA cells. Naïve SCs are believed to exist in low numbers and the high number of SSEA-1+ cells present human endometrium supports TA identity (almost all epithelial cells in the basal glands of cycling endometrium and PM tissue expressed SSEA-1). In addition, TA cells will divide for a restricted period of time: gland-like structures are incapable of surviving after 14 days in culture.[212] It is possible that a more potent and primitive epithelial ASC exists in human endometrium, one that possibly exists in the SSEA-1- fraction.

When looking specifically at the SSEA-1- population; OC, a marker of osteocyte differentiation was actually down-regulated. It was noted that *osteogenic media* favoured endometrial proliferation and there was a substantial increase in the number of cells. This was probably on account of the reagent vitamin D, which has been shown to increase proliferation of endometrial cells in culture.[213] The reduction in OC expression is likely to be relative to an increase in new endometrial cells. Cells cultured in adipogenic media showed marginal increases in PPARγ2 expression and

lipid vacuoles were present in one cell. It is unclear whether this cell was epithelial in origin. Data from our laboratory (not shown) has demonstrated that the epithelial fraction isolated from endometrial tissue has approximately 20% stromal contamination. As SSEA-1 is only expressed on epithelial cells, the SSEA-1+ fraction is likely to be 'purer' than its SSEA-1- counterpart. The findings of differentiation could be due to stroma or resident bone marrow cell contamination, both of which we know to be multipotent.[138-148,180] A pragmatic approach would be to suggest that an EEp-ASPC, irrespective of its place in the SC hierarchy, would be unipotent. This is similar to epithelial SPCs found in the skin, liver and pancreas.[212,214,215] These findings challenge the work of *Cervello et al.,* the authors of which claim endometrial epithelia are capable of mesodermal differentiation.[141,147] However, it is worth noting that epithelial cells used by this group were passaged up to 15 times: it is possible cellular phenotype changed in culture within this time period. Primary human epithelial cells are reputed to be fastidious and difficult to grow in culture; after passaging, primary epithelial cultures are often hijacked by contaminating stromal cells, which can withstand long term culturing. The study is also open to criticism on account of the morphology of differentiated cells. There is increasing evidence to suggest lipid vacuoles, which stain positive with Oil Red O, can be a response to stress.[216] Given the difficulty we experienced at trying to get epithelial cells to survive in adipogenic media, and the presence of Oil Red O staining in control cells too, we postulate this is the most likely cause of lipid vacuoles, rather than differentiation into adipocytes.

The unipotency of endometrial epithelial SPCs may also explain the pathophysiology of endometriosis. Current dogma suggests that endometriosis is a consequence of

retrograde menstruation of an endometrial SPC.[45,217,218] Data from our laboratory (not shown) has demonstrated that SSEA-1 expression is significantly greater in the functionalis of the endometrium during the secretory phase of women with endometriosis. Therefore, it is likely that a greater number of unipotent SSEA-1+ cells are thrust into the pelvic cavity via retrograde menstruation. Some lines of evidence imply there is an epithelial-mesenchyme relationship governing epithelial tissue cell fate. For example, central corneal epithelia can be reprogrammed to form pilosebaceous units when transplanted into dermis.[99] However, this does not occur in endometriosis and further supports the theory that EEp-ASPCs are committed.

A great deal of experimental research has tried to replicate SC niches in order to promote SPC differentiation *in vitro*. When looking specifically at human endometrium, perhaps we have been making things too difficult for ourselves. If we examine *in vivo* physiology, we know that human endometrium is in very close proximity to the most "pluripotent" niche of all - the developing blastocyst. It is this niche, which allows ESC differentiation into all cells types. Since human endometrial decidua does not differentiate into other cell types when exposed to the blastocyst, it would be irrational to assume it would differentiate into other cell types *in vitro.*

The commitment of endometrial SPCs to produce only endometrium may indeed be a design advantage. It is now well recognised that some cells are exchanged at the placental border between mother and foetus.[219,220] These microchimerisms (Mc) usually are foetal-maternal in direction, and have been shown to still be present in maternal tissue many years post-partum.[221] Conversely, maternal microchimerism

(MMc) is the passage of maternal progenitor cells into foetal tissue. This is less well researched and currently an area of active investigation. MMc tissue has been found in foetal thymus, liver, heart, pancreas and ovary.[222] It is postulated MMc occurs to promote foetal immunity. However, research remains inconclusive as to whether the trafficking of maternal cells in the foetus is beneficial or not. Despite MMcs being found in healthy foetal tissue, there is an increased association with disease in the progeny, such as Hirschprung's Disease, biliary atresia, type 1 diabetes, juvenile myositis and neonatal lupus.[223-227] Most cells transferred are haematopoietic SCs (CD34+), which have multipotent properties.[222] These findings together suggest the transfer of maternal multipotent SPCs into the foetal circulation could be hazardous. Therefore, given the close proximity of endometrial decidua to the zygote, it would be beneficial for endometrial SPCs to be restricted to unipotency capacity, to prevent damage to offspring.

In addition, in 1986, *Finn* proposed menstruation was the consequence of non-reversible endometrial differentiation, required to ensure implantation occurred at the optimum time.[18] Menstruation is a common cause of anaemia in pre-menopausal women; it could be regarded as a 'design flaw'. If *Finn* were correct, it would seem illogical for endometrial SPCs to have the capacity to cross lineages and produce other cell types when it cannot 'de-differentiate' into early proliferative endometrium.

It is also this author's belief that multipotency of ESt-ASPCs is questionable. Although there are numerous papers suggestive of differentiation into adipocytes, osteocytes and chondrocytes, the data can be interpreted as misleading; studies are

deficient, with experiments lacking basic controls.[138-148] Differentiation is a transitional process. Therefore, it necessitates parameters to be assessed 'before' and 'after' intervention, in this case - culture induction media. As conventional measurements, such as PCR and staining, require the fixation or ligation of cells, this is not possible in cell culture; the initial measurement would be at the expense of the final one. To get around this, stem cell biologists culture the same population of cells in induction and non-induction media. At the end of the time period, induction media is regarded as the 'after' measurement and non-induction is the 'before'. Therefore, to assess differentiation, both before and after measurements are *mandatory*. Unfortunately, current literature regarding the differentiation potential of endometrial ASPCs does not volunteer both of these parameters. Furthermore, as with any experiment, a positive control should be included. Although differentiation assays require analysis of gene expression and immuno-phenotyping, it is the differentiation *potential* that is really being tested, and therefore MSCs should be the positive control; changes in phenotype of studied ASPCs may only be marginal in comparison.

6.4: Human endometrium harbours an SPC with neurogenic potential

Noureddini et al. have demonstrated that cells sorted on CD146+ have the capacity to produce neural-like cells when prompted.[228] Other work studying endometrial stromal cells demonstrated similar effects.[151] To the best of our knowledge, this is the first study to investigate neural differentiation from endometrial epithelia. However, this work is preliminary and further work is required to validate findings. Nonetheless, there was an increase in PGP9.5+ cells when cultured in neurogenic media, compared with control. Neurons do exist in human endometrium and it would

be rational to accept there exists a neural progenitor within the tissue. However, it is unlikely SSEA-1+ and SSEA-1- cells trans-differentiated into neural cells, as they were unable to demonstrate multipotency in other assays. PGP9.5 is also a marker of neuroendocrine cells and non-small tumour cells in the lung.[229] In addition, *Quinn et al*., observed cross reactivity for PGP9.5 with endometrial glands when trying to investigate the presence of neural fibres in the endometrium.[15] No further details were divulged as to the location of the glands or staining pattern, as it was an incidental finding. It could be that PGP9.5 is present on endometrial glands normally; however it is still interesting that these cells proliferate in the neurogenic media, suggesting a neural-like phenotype. Ideally, other neural markers would be used along with a comprehensive immuno-histochemical (IHC) study of PGP9.5 in tissue sections, but unfortunately this was not possible within the time course of the project.

6.5: CK5/6 as a putative SPC for endometriosis

It was an interesting observation to discover non-gland-like structures in 3D media had strong staining intensity for CK5/6. It remains unclear as to what phenotype these cells are. An absence of β-catenin and CK18 would suggest stromal contamination. These cells would ideally be stained with a specific endometrial stromal marker to validate this. At present there appears to be no such thing, as data suggest vimentin is present in stroma but also mesodermal derived epithelia.[230] However, if cells *were* stromal, it is also curious as to why they should express CK5/6 *in vitro* but not *in vivo*. Similarly, the undefined cells demonstrated negative immuno-reactivity for all hormonal receptors, unlike *in vivo* stromal behaviour. It could be that these cells are luminal epithelia, which showed consistent strong

staining intensity for CK5/6 *in vivo*. Conversely, the fact these cells do not form a monolayer, resemble *in vivo* morphology or express hormone receptors, would counteract this argument.

It is noteworthy that these cells were only observed in culture samples from an endometriosis patient. Due to the small numbers of patients recruited it is difficult to elicit whether this is significant, but it does raise suspicion as to whether the cells are pathological. One theory may be Epithelial-Mesenchyme Transition (EMT) and Mesenchyme-Epithelia Transition (MET). The process of EMT and MET have both been implicated in the pathogenesis of endometriosis, and would explain why these cells demonstrated negative immuno-reactivity to epithelial markers but this theory warrants future work.[230]

It is possible that these undefined cells are representative of a SC phenotype. In breast, the CK5/6+CK18- phenotype has the ability to differentiate into other cells types.[231,232] We know the endometrium and breast are related; both undergo differentiation under the influence of oestrogen.

Perhaps these undefined structures are representative of an endometriosis stem cell. Counterarguments would include the negative immuno-reactivity for ER receptors. As endometriosis is dependent upon oestrogen it could be assumed an endometriosis SPC would have positive ER status. However, ER is believed to be involved in the maintenance of ectopic lesions, not necessarily the initiation of disease. Again, if we turn our attention to the breast; a high H-score for CK5/6+ cells in breast carcinoma was associated with a higher tumour grade, recurrence, metastasis and low ER

immuno-reactivity.[232] Together, these findings support the need for further work investigating CK5/6 and its role in endometrial physiology and pathology.

6.6: Limitations

Sample numbers within the study are noticeably small and caused some results to be statistically insignificant. Unfortunately, this is a common consequence of primary cell culture. Preferably numbers would be higher; it is unknown how this could be improved as the study did take place at a tertiary centre for gynaecology, arguably the largest women's specialist hospital in Western Europe. A real obstacle was the acquisition of endometriosis samples, as high numbers had to be excluded from the study. Patients were recruited at the time of surgery, and subsequently most had received hormonal therapy prior to this time. It is also debatable how much the 'control' samples are non-pathological. Patients underwent surgery for menorrhagia, benign polyps and pelvic pain. It is possible that these patients may have aberrant SPC behaviour that is not representative of physiologic norms. Since completely healthy women do not have hysterectomies or undergo investigative procedures, it is difficult to access completely healthy endometrium.

IHC analysis of gland-like structures would ideally be scored and compared with *in vivo* tissue. Low numbers of 3D structures meant this was not possible. In addition, it is not known whether IHC and antibodies penetrate the organoids in the same way as tissue sections; it was noted gland-like structures had greater staining intensity for haematoxylin when compared to tissue sections, despite being incubated for the same time.

When discussing SCs, in theory these should represent a small sub-population of cells. Stromal contamination was estimated to be roughly 20%. Because of this, combined with suggestions that stromal cells are multipotent, it is difficult to establish whether the few cells that have differentiated into other cell types are epithelial in origin. A way to overcome this would be to dual sort by FACS on CD9+/CD15+. However, from our experience FACs has a significant effect on endometrial cell viability and these observations are supported by others.[163,164,190]

Data for neural differentiation is preliminary; one marker alone has verified results via only one assay. Ideally, a panel of neural markers and qPCR would be performed against a positive control to establish how 'successful' this differentiation is. Unfortunately within the course of the project this was not possible. In respect of this, there is a huge difficulty within the SC field to establish whether a cell has differentiated successfully or whether it has merely adapted to advantageous/disadvantageous surroundings. As it stands, most scientists confirm successful mesodermal differentiation by one staining method and RT-PCR of two markers. However, some lines of evidence suggest these methods are not definitive.[216] The ultimate test, requires a quantitative *in vivo* assay to confirm 'differentiated' cells are functional for each cell type, and currently this is not available.

6.7: Future Work

Gland-like structures cultured in 3D media best resemble PM glands on account of an inert hormonal environment. We can hypothesise that the introduction of exogenous hormones to mimic the menstrual cycle would reproduce the

morphological changes associated with cycling endometrial tissue, and this is an area of future development. A greater understanding of endometrial stromal cells in 3D culture is also warranted, in the hope of developing a 3D co-culture model that produces endometrial tissue as a whole. The next step ultimately would be to investigate the behaviour of these cells *in vivo* using an animal model.

The suggestion of a neural pre-cursor in human endometrium raises many questions. Eutopic endometrium from endometriosis patients is found to have increased neural fibres, and there is a hyper-algesic element to the disease.[60] It would be interesting to assess if there are differences in endometrial neural progenitor activity in eutopic endometrium from endometriosis patients when compared to controls.

Results regarding CK5/6 staining in 3D media are interesting but remain inconclusive. There are several theories regarding the presence of these otherwise undefined cells and each one of them needs to be explored. The first aim would be to reproduce results by increasing patient numbers. Secondly, culture of stromal cells and IHC analysis are required to establish whether mesenchyme begin to express CK5/6 in a 3D environment and if so, why. Similarly, luminal epithelia should be isolated and cultured in the same environment to establish whether these cells are responsible for non-defined structures. At present, unlike other mucosal tissue such as breast, intestine and skin, there is no marker that discriminates *between endometrial* epithelia. qPCR analysis on CK5/6+ cells verses CK5/6- could be implemented to investigate whether there is any increased expression of stem cell markers within the positive population. This could also be compared between normal and endometriosis samples.

Irrespective of stem cells, it is important to note that CK5/6 only stains some and not all glandular epithelia *in vivo.* This warrants the question as to whether there are different epithelia sub-types. A full understanding of differentiated endometrial epithelia is necessary to appreciate SPC behaviour. Furthermore, the amount of research investigating changes in cell behaviour throughout the menstrual cycle and between functional and basal layers remains sparse. When one acknowledges the endometrium's regenerative abilities, and the clear distinction between old and new tissue, it becomes apparent that this highly dynamic tissue is being overlooked. It could be the ideal platform to study stem cell behaviour.

6.8: Conclusion

There is growing evidence to suggest that the endometrium harbours an ASPC population. We have validated our previous work and established that SSEA-1+ cells have a greater propensity to produce gland-like structures that resemble glands in PM tissue, confirming ASPC status. When the same cells were encouraged to differentiate into other cell types from the mesoderm results were disappointing, suggesting that SSEA-1+ ASPCs are unipotent. This work agrees with the dogma that endometriosis is due to the retrograde menstruation of a unipotent ASPC. Interesting, both SSEA-1+ and SSEA-1- cell populations were able to produce an increase in neural-like cells when cultured in appropriate induction media. It is unclear whether this is trans-differentiation or merely that neurogenic media facilitates growth of a neuroendocrine cell phenotype. Nonetheless, it may have ramifications in the development of hyper-algesic diseases such as endometriosis. 3D culture of eutopic endometriosis endometrium produces non-gland-like structures,

which express CK5/6 and may have some implications into the development of the disease. A greater understanding of endometrial epithelial composition and behaviour throughout the cycle is necessary to appreciate epithelial SPC behaviour. Stem cell research is a difficult area of study, as theories and definitions remain blurred and divide opinion. It is hoped that this work provides a stepping-stone to understanding endometrial epithelial ASPC behaviour in health and disease.

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APPENDIX-I

National Research Ethics Service
North West 2 Research Ethics Committee - Liverpool Central
Room 181
Gateway House
Piccadilly South
Manchester
M60 7LP

Telephone: 0161 237 2336
Facsimile: 0161 237 2383

05 October 2009

Dr Dharani K Hapangama
Clinical Senior Lecturer / Honorory Consultant in Obstetrics & Gynaecology
University of Liverpool
University Department, First Floor
Liverpool Women's Hospital,Crown St
Liverpool
L8 7SS

Dear Dr Hapangama

Study Title:	**The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis**
REC reference number:	**09/H1005/55**
Protocol number:	**1.0**

Thank you for your letter of 14 September 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a sub-committee of the REC. A list of the sub-committee members is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research

governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. *Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.*

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		
REC application	2.2	
Protocol	1.0	02 July 2009
Investigator CV		
GP/Consultant Information Sheets		
Letter from Sponsor		02 July 2009
Referees or other scientific critique report		
Participant Information Sheet	2	09 September 2009
Participant Consent Form	2	09 September 2009
poster healthy + baby		
poster endometriosis		
Response to Request for Further Information		14 September 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of

changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H1005/55	**Please quote this number on all correspondence**

Yours sincerely

Professor Sobhan Vinjamuri
Chair

Email: carol.ebenezer@northwest.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

"After ethical review – guidance for researchers"

Copy to: *Mrs Gillian Vernon*

Liverpool Women's
NHS Foundation Trust

Ethics Submission No: 09/H1005/55
PATIENT INFORMATION SHEET

"Endometrial stem cell Study"

The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis.

Version 1: Endometrial biopsy only

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this

Why are we doing the study?

The inner lining of the womb (endometrium) may play an important part in endometriosis. **Endometriosis** is a common condition in which patches of the inner lining of the womb appear in parts of the body other than the cavity of the womb and seen in 1 in 10 women below the age of 50. It can cause painful periods, pelvic pain, pain with sexual intercourse and infertility. It is possible that an abnormality of specials cells in the endometrium called stem cells can cause endometriosis. If so, the information from this study will help us to develop new techniques to diagnose and treat this distressing condition.

What are stem cells?

Stem cells are special cells that can renew themselves (adult stem cells), and their job in the body is not yet determined. The inner-lining of the womb (endometrium) has these stem cells that can become many different types of cells, and they are likely to be responsible for its monthly regeneration. With monthly bleeding these cells are shed and can be expelled into the abdominal cavity. If these cells are implanted in the pelvis they can cause endometriosis as endometriosis occurs when endometrial cells of are found growing outside of the womb. We believe that abnormalities of these stem cells may cause endometriosis.

Why have I been chosen?

We are looking for a total of 160 women (you must have been off all hormonal medicines for at least 3 months), who have regular periods. We are specifically looking for 80 women who have endometriosis and another 80 completely healthy women who have had at least one baby. If you belong to any of these groups we will ask you if you would want to take part in the study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

1. *If you are having a hysterectomy:*
   Your operation will continue exactly as planned. However, once the operation is over, a small sample of the womb that has already been removed will be taken for the research.
2. *If you are not having a hysterectomy:*
   Your operation will continue exactly as planned. However, a pipelle sample (see below) will also be taken from you for research.

The sample of endometrium will be processed in the lab to isolate the stem cells from it. Therefore, **NO** extra surgery will be performed for the study. A **blood sample** (5mls = teaspoonful of) will also be taken from your veins.

How is the endometrial pipelle sample done?

Whilst you are in the clinic (without anaesthetic) or whilst you are under anesthetic, the doctor will place a speculum (just like when you have a cervical smear) in the vagina. A plastic instrument (like a blunt drinking straw) will then be introduced through the neck of your womb to gently suck some cells from the inner lining of the womb. These cells will be then sent to the laboratory to be examined. This procedure is routinely done in our Gynaecology clinic and apart from the mild lower abdominal period like discomfort and vaginal spotting, it does not usually cause other problems. If you are going to have the biopsy taken at the time of the operation under anesthetic, you will not have any extra discomfort.

What are the possible benefits of taking part?

We do not expect you to have any extra benefits by taking part in this study. However, you will be helping us to improve our knowledge about endometriosis.

What if something goes wrong?

There are no special compensation arrangements in place to compensate you in case if taking part in this research project harms you. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Data management

The information gathered from this study will be entered into a computer database and will be analyzed in a strictly confidential manner, in compliance with the Data Protection Act. Regulatory authorities for approving medicines and the University of Liverpool may wish to look at medical records to check that the study has been performed correctly. All information, which is collected about you during the course of the research will be kept

strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it. Once we carryout the study on the samples you kindly donate, if there is any surplus tissue, it will be stored in the department of obstetrics & Gyanecology and will be used in other ethically approved studies.

If you are interested in taking part, please contact Dr. Dharani Hapangama (0151- 702 4114 or 0151 706 9988) in the Liverpool Women's Hospital, Crown Street, Liverpool.

If you want to find out more about the study from someone who is not directly involved in it and can give you unbiased advice, please contact Mr Jonathan Herod, Consultant, in Gynaecology Out Patient Clinic, telephone no. 0151 708 9988.

Liverpool
Women's
Hospital

Study Number:
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: **The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis.**

Name of Researcher: Dr Dharani Hapangama, Senior Lecturer
University of Liverpool / Liverpool Women's Hospital

Please initial box

1. I confirm that I have read and understand the information sheet dated ..... (version .....) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [University of Liverpool & Liverpool Women's Hospital] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study and for my GP to be informed of my part taking. ☐
5. I agree for surplus tissue to be stored in the department of obstetrics & Gyanecology and to be used in other ethically approved studies. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

Liverpool Women's
NHS Foundation Trust

Study number: LWH0813
Patient Identification Number for this trial:

Title of Project: The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis.

Researcher: Dr Dharani Hapangama
Clinical Senior Lecturer / Consultant in Obstetrics & Gynaecology Division of Perinatal and Reproductive Medicine School of Reproductive and Developmental Medicine First Floor Liverpool Women's Hospital Crown Street Liverpool L8 7SS

Age:

Height (cm):
Weight (kg):
Body mass index:

Smoking history:

Parity:
Miscarriage:
TOP:

Days of bleeding:
Cycle length:
LMP:

Endometriosis stage:

APPENDIX –II

STANDARD OPERATING PROCEDURE

Number	04	**Version & Issue**	1.2
Title	**TISSUE PROCESSING**		
Author	Jo Drury & Lisa Heathcote	**Date**	15/12/2006
Approved	Dr Siobhan Quenby	**Date**	16/12/2006
Review Date	October 2012	**Last Reviewed**	October 2011

BACKGROUND

Formalin fixed tissue is dehydrated, cleared then impregnated with paraffin wax using the automated Shandon Citadel 1000 processing machine. The processing setting for most sample types is ***programme A*** which operates using the schedule provided below. The total processing time is 18 ¾ hours, therefore the processor is generally run overnight. Starting the processor at 2pm will result in completion of the cycle at 8:45am the following morning.

Programme A Processing Schedule:

4% formalin in neutral buffer	45 minutes
60% Ethanol	1 hour
70% Ethanol	1 hour
90% Ethanol	1 hour
100% Ethanol	1 hour
100% Ethanol	1 ½ hours
100% Ethanol	2 hours
Xylene 1	1 hour
Xylene 2	1 ½ hours
Xylene 3	2 hours
Wax 1	2 ½ hours
Wax 2	3 ½ hours

EQUIPMENT INFORMATION:

Citadel processor (serial number CA 1390 EO 608) manufacturer: Thermo Electron Corporation. Manufacturer's address: 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England.

MAINTENANCE (CONTRACT AND SERVICING INFORMATION)

Maintained/serviced by Thermo Fisher Scientific 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England. Tel: 01928 562 541, Fax: 01928 562 512.

SOLVENT INFORMATION:

Ethanol and xylene are purchased from Chemistry solvent stores (University of Liverpool). The wax (Histoplast PE REF8330) is purchased from Thermo Scientific, 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England. The 10% neutral buffered formalin is purchased from Sigma Life science, Fancy Road, Poole, Dorset, BH12 4QH.

HEALTH AND SAFETY PRECAUTIONS:

Wear a laboratory coat, safety spectacles and nitrile gloves. Xylene and formalin are harmful by contact to skin and eyes or if ingested or inhaled. Refer to the risk assessments and COSHH forms before starting procedure.

Overnight processing procedures require the completion of permits provided in safety circular SCR14/3 (link to document provided below). A 'Yellow permit' (for long term operation) should be prepared by the DSC or laboratory manager and the copy secured in a clear plastic folder on the door outside laboratory 4. The 'Pink' permit (valid for the duration of the experiment only) should be prepared by the processor user and the document should be placed on or adjacent to the apparatus. Information that needs to be recorded on the pink copy includes:

a) experimental details

b) times and dates of experiment

c) emergency procedures

d) name, address and telephone number of the processor user

e) signature of researcher concerned and a countersignature from the laboratory manager/DSC.

PROTOCOL

A Preparation of Solvent Containers on Processing Machine:

Between processing runs, ethanol solutions are stored in labelled bottles to prevent evaporation. These are found in the metal solvent cabinet under the hood in Laboratory 4. Therefore, the solvent containers must be re-filled before tissue processing

commences. To access the processor solvent container, press the ***raise*** button on the hand held controller***.*** Remove the evaporation covers from the top of the processor then press ***rotate*** or ***check/fill*** button. The rotate function will cause the operating head assembly to advance one position and the check/fill option rotates the head three positions. Remove the container using the metal handles and add 1.5L of the required ethanol solution (the top of the bevelled edge on the solvent container is approximately 1.5L). Repeat the process until all ethanol containers are filled. It is also advisable to check the level of the xylene, formalin and the wax containers on a regular basis. If fresh 100% ethanol is used to top up or prepare solutions for the processor ensure the ethanol log is updated with the volume used. The ethanol log is situated in DSC office (room 1128).

B Sample Processing Procedure

1. Label plastic cassettes with the sample ID number using pencil.
2. Carefully remove the tissue from the formalin pot using forceps and place into the cassette. It is advisable to use small wire inserts if the tissue is in small fragments, and a larger yellow cassette if the tissue pieces are substantial. Secure the inserts into the plastic cassettes by snapping closed the lid and re-check the sample ID. Store the filled cassettes in a Petri dish of formalin until they are all ready to be placed into the processor. Dispose of the used formalin in the red labelled waste container which is stored below the fume hood.
2. Arrange the cassettes so they are loosely spaced in the processing baskets. There are 3 different sizes of basket – use the smallest one required to contain all the cassettes.
3. Press ***Go To 1*** on the hand held controller**,** slot the processing baskets onto the holder over the formalin and place weight on top of the basket.
4. Press ***Lower*** button on the hand held controller (to lower the basket into the formalin).
5. Check the time is correct by pressing ***clock*** (alter time by pressing the + button or whilst holding in the clock button if necessary).
6. Select the appropriate processing time function:

Date of Processing	Processing Time	Hand Held Controller Action
Monday-Thursday	Before 2pm	Press ***delay on*** button
Monday-Thursday	After 2pm	Press ***autostart*** button
Friday	Before 2pm	Press ***days delay,*** hold this in, scroll to 2 days using the + button then press ***delay on.***
Friday	After 2pm	press ***days delay,*** hold this in, scroll to 1 day using the + button, then press ***delay on***

7. Complete the Citadel 1000 user log (the red folder is situated by the embedding station in laboratory 4) with sample, solvent and programme information.
8. When the processing cycles are completed, switch on the Shandon Histocentre 3 embedding machine.
9. Press ***raise*** button on the hand held controller and remove baskets from holder.
   Use paper towels to stop wax being dripped onto the floor.
10. Place blocks into heated reservoir in the embedding machine.
11. Wipe processing baskets with absorbent towels to remove surplus wax then place equipment into the oven (heated to ~100°C) for several minutes to remove any residue. Ensure the rubber seal is removed from the basket holder before it is put into the oven.
12. Soak wire inserts in xylene for several hours/overnight to remove wax residues. Leave the inserts in the fume hood to air dry.
13. Remove the alcohols from the processing machine using the method described in section A and pour solutions into the labelled storage bottles using a funnel. The storage bottles are then placed back in the flammable solvent metal storage cabinets.
14. Update the Citadel 1000 processing user log.

C Disposal of Waste Solvents

The solvents must be changed on a regular basis (depending on time of year and processor usage) to ensure efficient processing of the tissue samples. Waste solvents must be placed in the red solvent waste containers and identified clearly with the appropriate labels provided by Lisa Heathcote or Jo Drury. Contaminated waste wax can be discarded by pouring the molten solution into a container and cooling until the wax is

set. The wax container can then be disposed of in the LWH clinical waste. Refer to the waste disposal protocol (SOP 25) for further information.

Other useful SOPs associated with 04

Embedding samples using the Shandon Histocentre 3 machine (SOP 05).
Processing of endometrial biopsy samples (SOP 03).
Disposal of solvent waste (SOP 25).

SOP History

Original SOP prepared by J Drury and L Heathcote in December 2006.

Version 1.1 was prepared to include changes in both Liverpool Women's Hospital and University of Liverpool new logos. In addition, supplier changes for consumables information have been updated.

Version 1.2 was prepared to include the latest safety circular information, biopsy insert dewaxing procedure and unattended experiment information.

Appendices - Associated Documents

	Document	Location
1	COSHH form for processing samples	COSHH H&S folder
2	Risk assessment form for human samples	Risk assessment folder
3	Safety circular on waste disposal	Safety circular folder room 1128 or downloaded from UoL safety website .
4	Unattended experiments outside normal working hours safety circular SCR14/3	Safety circular folder room 1128 or downloaded from UoL safety website (http://www.liv.ac.uk/safety/documents_guidance/Safety%20circulars/Safety_Circular_SCR14.pdf).

Review Date: October 2012

STANDARD OPERATING PROCEDURE

Number	**07**	**Version & Issue**	1.2
Title	**APES coating procedure**		
Author	Jo Drury & Lisa Heathcote	**Date**	21/5/2007
Approved	Dr Siobhan Quenby	**Date**	23/5/2007
Review Date	October 2012	**Last Reviewed**	October 2011

BACKGROUND

The Aminopropyl triethoxy silane (APES) coating procedure is used to improve tissue adhesion by producing a positive charge on the surface of the slide. The protocol described below was modified from the original method described by Maddox and Jenkins in 1991 (full reference provided in the appendices).

HEALTH AND SAFETY REQUIREMENTS

The APES concentrate is a toxic chemical that is moisture sensitive and requires storage at 0-5°C; it is an irritant and can cause burns. Normal laboratory precautions, including the wearing of gloves and the use of a fume cupboard, should be taken when handling the concentrate solution. Refer to the risk assessments and COSHH forms before starting procedure. Place all waste APES solution and ethanol into labelled red solvent waste cans and attach a solvent label (available from Lisa Heathcote or Jo Drury by request). Disposal of all solvents (ethanol and ethanol/APES mix) is via Solvent Services (solvents@liv.ac.uk).

EQUIPMENT INFORMATION:

- Microscope slides (twin frost size 26 x 76mm-Printed “IVD CE” 90º ground edges, catalogue number MAE-1000-03P Pack of 1000) are purchased from Liverpool Women’s Hospital (NHS) purchasing department.
- APES solution (catalogue number A3648) is purchased from Sigma-Aldrich Company Ltd. UK distributor address: The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT.
- Staining racks and glass jars are supplied by Raymond A Lamb Ltd. Manufacturer’s address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.

METHOD:

Work in the fume hood

1. Place slides into plastic racks. Each rack holds 24 slides.
2. Prepare a 2% APES working solution by mixing 5ml APES with 245ml 100% ethanol, or for the bulk method 40ml APES with 1960ml 100% ethanol. The APES solution is stable for several hours; therefore coat as many racks as possible to save reagents and time.
3. Prepare a glass staining dish containing 100% ethanol.

Position 1: APES working solution

Positions 2 – 4: distilled water

4. Place slide racks in 100% ethanol. Dip several times to dislodge dust etc., and then drain the staining racks on absorbent paper towels.
5. Incubate the slides for 5 minutes in APES working solution.
6. Rinse slides in three changes of distilled water (2 minutes incubation for each rinse).
7. Drain slides on absorbent towels and air dry overnight, or dry in a section dryer for a few hours at 37°C.
8. Discard the first change of distilled water. Place the staining dish containing fresh distilled water at the last rinse location (position 4). Therefore the second change of water has becomes the first etc.
9. Repeat steps 5 through to 8 for each rack of slides. Allow to dry at room temperature or in the drying oven at 37 °C.
10. Transfer the coated slides to a dust free container labelled ‘APES’ and the date of coating (APES slides are stable for approximately 3 months).

BULK METHOD

This is the same method as above, but on a larger scale. Use large plastic trays to contain the APES solution and distilled water. As this method uses large quantities of reagents, it should only be used when time is limited.

Other useful SOPs associated with 07 Procedure

ImmPress immunohistochemical procedure (SOP 12).

Cutting paraffin sections (SOP 06)

SOP History

Original SOP prepared by J Drury and L Heathcote in

This version was prepared to include changes in both Liverpool Women's Hospital and University of Liverpool new logos.

Version 1.2 was prepared to include the Bulk method and new solvent disposal information.

Appendices - Associated Documents

	Document	**Location**
1	COSHH assessment for APES coating	COSHH assessment folder in the deputy departmental safety advisors office
2	Disposal safety circular	Health and safety intranet on the University of Liverpool web page
3.	P. H. Maddox and D. Jenkins (1987) 3-aminopropyltriethoxysilane (APES): a new advance in section adhesion J Clin Pathol 40:1256 - 1260 3 G. Niedobitek and H. Herbst (1991).	Paper copies available from Lisa Heathcote by request.

Review Date: October 2012

STANDARD OPERATING PROCEDURE

Number	13	**Version & Issue**	1.1
Title	IMMUNOHISTOCHEMISTRY: HAEMATOXYLIN AND EOSIN STAINING OF BIOPSY TISSUE SAMPLES		
Author	Jo Drury & Lisa Heathcote	**Date**	15/12/2006
Approved	Dr Siobhan Quenby	**Date**	TBC
Review Date	October 2012	**Last Reviewed**	October 2011

BACKGROUND

The Haematoxylin and Eosin stain is probably the most widely used stain in Histopathology/ Cytopathology. It has the ability to demonstrate an enormous amount of tissue structures. The haematoxylin component stains cell nuclei blue / black with good intranuclear detail whilst the eosin stains cell cytoplasm and most connective tissue fibres in varying shades of pink.

Health and Safety Precautions

The main health and safety risks to this procedure arise from chemicals/reagents that are potentially hazardous, gloves must be worn at all times. Eosin Y aqueous contains (formaldehyde: 50 – 00 – 0), causes burns, and may cause sensitisation by skin contact. In addition, it is toxic by inhalation, in contact with skin and if swallowed. Haematoxylin (Gill 2) contains (ethylene glycol: 107 – 21 – 1; aluminium sulphate 10043 – 01 – 3; acetic acid: 64 – 19 – 7). This is harmful if swallowed; very toxic by inhalation; causes severe burns, and is irritating to eyes.

METHOD

Work in a fume hood. Rack slides, bake (see SOP 08 for more details) and use long forceps to transfer the rack from dish to dish. Drain off excess liquid from the slide rack on paper towels before transfer to the next dish.

For paraffin sections:

1. Dewax prior to staining: 10 min Xylene 1
   10 min Xylene 2
   5 min 100% Ethanol 1
   5 min 100% Ethanol 2

1 min 90% Ethanol

1 min 70% Ethanol

2. 2 min water

3. Gills 2 Haematoxylin (1 min 30s – 2 min)

4. Rinse in running tap water until water runs clear

5. Acid alcohol dip (very briefly)

6. Blue in running tap water (approximately 5 min)

7. 70% alcohol 30s – 1 min

8. 95% alcohol 30s – 1 min

9. Eosin Y, aqueous 4 min

10. Water rinse x 2

11. Incubate water 2 min

12. 95% alcohol 20 - 30s

13. 100% alcohol 1: 30s – 1 min

14. 100% alcohol 2: 30s – 1 min

15. Xylene 1: 5mins

16. Xylene 2: 10mins

17. DPX and mount

Other useful SOPs associated with 13

Reception and processing of endometrial biopsies (SOP 03).

Embedding samples using the Shandon Histocentre 3 (SOP 05).

Cutting paraffin sections (SOP 06)

Dewaxing paraffin sections for staining (SOP 08)

Disposal of solvent waste (SOP 25).

SOP History

Original SOP prepared by J Drury and L Heathcote in December 2006.

This version was prepared to include changes in both Liverpool Women's Hospital and University of Liverpool new logos.

Appendices -Associated Documents

	Document	Location
1	Risk and COSHH assessments	Health and Safety folders in the departmental safety advisors office
2		

Review Date: October 2012

APPENDIX – III

	GLAND-LIKE STRUCTURES					PM GLANDS				
	+ve	Σ	%	Mean	SD	+ve	Σ	%	Mean	SD
	11	16	68.8%			28	35	80.0%		
	14	19	73.7%			29	31	93.5%		
	15	18	83.3%			44	51	86.3%		
	26	33	78.8%			56	68	82.4%		
	85	86	98.8%			51	56	91.1%		
	23	23	100.0%			14	22	63.6%		
	54	57	94.7%			27	31	87.1%		
	43	50	86.0%			64	76	84.2%		
	45	47	95.7%			36	46	78.3%		
	54	57	94.7%			17	27	63.0%		
	87	98	88.8%			25	28	89.3%	81.70%	10.17%
	29	32	90.6%							
	24	27	88.9%							
	60	69	87.0%							
	76	84	90.5%							
	36	39	92.3%							
	13	13	100.0%							
ER-α	38	46	82.6%	88.62%	8.74%					
	14	15	93.33%			116	116	100.00%		
	65	67	97.01%			53	53	100.00%		
	63	64	98.44%			36	36	100.00%		
	41	43	95.35%			35	35	100.00%		
	70	76	92.11%			48	48	100.00%		
	31	34	91.18%			75	75	100.00%		
	28	28	100.00%			66	68	97.06%		
	20	20	100.00%			31	31	100.00%		
	38	42	90.48%			36	36	100.00%		
	13	13	100.00%			18	18	100.00%	99.71%	0.93%
	18	18	100.00%							
	14	17	82.35%							
ER-β	36	41	87.80%	94.47%	5.55%					
	2	20	10.00%			29	29	100.00%		
	7	23	30.43%			32	42	76.19%		
	6	20	30.00%			22	29	75.86%		
	10	28	35.71%			60	63	95.24%		
	36	80	45.00%			60	69	86.96%		
	18	55	32.73%			76	103	73.79%		
	28	75	37.33%			47	54	87.04%		
	22	64	34.38%			31	42	73.81%		
	22	64	34.38%			66	77	85.71%		
	8	46	17.39%			50	60	83.33%	83.79%	9.06%
	7	21	33.33%							
	6	17	35.29%							
	10	48	20.83%							
	16	37	43.24%							
PR	7	19	36.84%	31.79%	9.31%					

		+ve	Σ	%	Mean	SD
		5	13	38.46%		
		6	19	31.58%		
		7	22	31.82%		
		3	17	17.65%		
		7	22	31.82%		
		6	15	40.00%		
		5	14	35.71%		
		5	16	31.25%		
		4	13	30.77%		
	Neuro+	6	24	25.00%	31.41%	6.44%
		3	28	10.71%		
		3	25	12.00%		
		3	22	13.64%		
		2	31	6.45%		
		5	34	14.71%		
		4	27	14.81%		
		3	27	11.11%		
		4	26	15.38%		
		6	32	18.75%		
14+	C+	3	20	15.00%	13.26%	3.36%
		6	13	46.15%		
		6	16	37.50%		
		3	14	21.43%		
		10	36	27.78%		
		12	28	42.86%		
		12	39	30.77%		
		3	11	27.27%		
		7	25	28.00%		
		10	37	27.03%		
	Neuro-	14	28	50.00%	33.88%	9.61%
		2	24	8.33%		
		3	23	13.04%		
		4	28	14.29%		
		3	24	12.50%		
		2	27	7.41%		
		2	20	10.00%		
		2	31	6.45%		
		4	27	14.81%		
		2	28	7.14%		
14-	C-	3	22	13.64%	10.76%	3.24%

		+ve	Σ	%	Mean	SD
		14	41	34.15%		
		9	42	21.43%		
		14	42	33.33%		
		16	38	42.11%		
		13	42	30.95%		
		7	30	23.33%		
		3	23	13.04%		
		7	20	35.00%		
		/	/			
	Neuro+	9	21	42.86%	30.69%	9.26%
		4	19	21.05%		
		2	14	14.29%		
		4	22	18.18%		
		2	27	7.41%		
		2	22	9.09%		
		1	27	3.70%		
		2	25	8.00%		
		2	20	10.00%		
		2	14	14.29%		
20+	C+	3	32	9.38%	11.54%	5.31%
		7	34	20.59%		
		10	66	15.15%		
		10	50	20.00%		
		13	55	23.64%		
		12	47	25.53%		
		15	49	30.61%		
		14	51	27.45%		
		14	46	30.43%		
		13	47	27.66%		
	Neuro-	15	54	27.78%	24.88%	5.00%
		4	44	9.09%		
		3	40	7.50%		
		4	32	12.50%		
		3	53	5.66%		
		4	59	6.78%		
		4	40	10.00%		
		4	35	11.43%		
		2	32	6.25%		
		1	31	3.23%		
20-	C-	1	39	2.56%	7.50%	3.29%

		+ve	Σ	%	Mean	SD
		2	14	14.29%		
		2	17	11.76%		
		4	25	16.00%		
		1	15	6.67%		
		1	6	16.67%		
		3	11	27.27%		
		6	19	31.58%		
		5	21	23.81%		
		2	14	14.29%		
	Neuro+	4	14	28.57%	19.09%	8.20%
		1	22	4.55%		
		2	19	10.53%		
		1	17	5.88%		
		1	21	4.76%		
		1	16	6.25%		
		2	34	5.88%		
		3	19	15.79%		
		3	24	12.50%		
		3	28	10.71%		
21+	C+	3	19	15.79%	9.26%	4.39%
		9	22	40.91%		
		5	20	25.00%		
		7	22	31.82%		
		11	37	29.73%		
		8	24	33.33%		
		15	42	35.71%		
		6	26	23.08%		
		8	37	21.62%		
		7	21	33.33%		
	Neuro-	9	24	37.50%	31.20%	6.34%
		2	27	7.41%		
		4	48	8.33%		
		4	41	9.76%		
		5	57	8.77%		
		4	25	16.00%		
		6	38	15.79%		
		5	42	11.90%		
		6	37	16.22%		
		5	40	12.50%		
21-	C-	6	47	12.77%	11.94%	3.32%